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ENDOCRINOLOGY

VOLUME 51 OCTOBER, 1952 NUMBER 4

HEPATIC BASOPHIL BODIES AND CANALICULI OF THE FROG IN TESTOSTERONE[1] ANAESTHESIA[2]

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THE action of the liver upon circulating steroid hormone has been investigated extensively, with reports continuing from the early work of Evans and Burr (1926), Golden and Severinghaus (1938), Talbot (1939) and others through the recent studies of Vanderlind and Westerfield (1950) and Grayhack and Scott (1951). Morphological effects of hormone upon the liver have been examined less frequently, but make up a substantial body of literature.

Among these, that of Korenchevsky (1941) is notable for its positive results. In a large series of experiments upon rats, he found that the basophilic granulations of the hepatic cells were depleted after gonadectomy and restored to normal number and size after administration of sex hormones. Similar investigations of liver structure after hormone treatment have been reported by Selye (1939), Mannerfelt (1947), Blackman (1944) and associates, and others; with no findings comparable to those of Korenchevsky.

In these investigations, small doses of hormone have been employed. The report of Selye (1941), of the anesthetic effect of large doses of steroid hormone, in rats, and the studies of Langan (1947, 1950), of similar action in frogs, suggested the use of such anaesthetic dosage in examination of the structure of the liver after hormone treatment. The demonstration by Knisely (1948) of the close similarity, in structure and function, of frog and mammalian liver, led to the selection of frogs as experimental animals.

Received for publication February 6, 1952.

[1] Hormone supplied by The Upjohn Company, through the kindness of M. H. Kuizenga and W. A. Iervolino.

[2] Includes the major content of a Ph.D. thesis presented at New York University.

Their large cells facilitated observation of tissue details and their small body weight permitted high body concentration of hormone with use of small quantities of material. Preliminary reports of this work have been published by Nace (1951, 1952). Some of the histological methods used, and the integration of the ribonucleic acid literature with other liver investigations, derive from the report of Lagerstedt (1949).

MATERIALS AND METHODS

Fifty female frogs, *Rana pipiens*, were obtained from Vermont. They had been collected in the spring and reached the laboratory in July. Body weights ranged from 26 gms. to 51 gms., with most animals falling between 30 and 40 gms.

The animals were maintained without food for six weeks before hormone injection, in order to eliminate periodic variation in liver activity, associated with digestion, and to permit a long period of treatment, of some animals, with possible liver poisons.

Twenty-two animals were treated with hormone. An equal number were used as controls. Each group included animals of similar size and weight.

The hormone employed was free testosterone, Upjohn Lot #T-24. It was administered intraperitoneally, in a 1% aqueous suspension containing 5% gum acacia.[3] This vehicle was chosen in order to avoid the effects of oil upon the liver, found in preliminary investigations.[4] The vacuoles found after oil treatment changed the positions of nuclei and basophil bodies. No effect upon number or size of basophil bodies was seen, nor did the bile canaliculi appear affected. But the spatial rearrangement of structures following sesame oil and peanut oil introduced an additional variable whose elimination appeared advantageous.

Hormone dosages ranged from 10 mg. to 80 mg., with most animals receiving approximately 1 mg. of hormone per gm. of body weight. The details of weight and dosage of each animal are given in Table I. In the same table are shown the time required for the loss of the righting reflex and for the development of deep anaesthesia, the time required for recovery from anaesthesia, and the time elapsed between injection of hormone and fixation of the liver.

One group of control animals was sacrificed without treatment. One control group received intraperitoneal injections of 5% gum acacia equal in volume to the hormone suspension administered to experimental animals of similar body weight. A third control group was anaesthetized with carbon tetrachloride vapor. Some carbon tetrachloride treated animals received hormone as well. Other animals, some of which received hormone, were treated repeatedly with tannic acid, phosphorus, Trypan Blue or India Ink, to provide samples of chemically injured livers.

After injection, the animals were observed closely for 24 hrs. At hourly intervals, they were examined for persistence of the righting reflex and for signs of deep anaesthesia, loss of respiratory movements and depression of heart action. At the time indicated in Table I, they were sacrificed and dissected by a standardized procedure. One lobe of each liver was fixed in Bouin's fluid. The rest of the liver, together with the heart, lungs and gall bladder, were fixed in Susa fluid and separated after fixation.

After 18 hrs. fixation, tissues were dehydrated in alcohol and benzene and imbedded

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[3] Suspension prepared by W. Florin, of Micro-Essential Laboratories.

[4] The tissues studied were provided by W. B. Langan, from his series of pharmacological experiments with a number of steroids. His reports are to be published elsewhere.

TABLE 1. DOSAGES, RESPONSE TIMES AND FIXATION TIMES OF EXPERIMENTAL ANIMALS

Body weight (Gms.)	Hormone dosage (Mgms.)	Hours to Stage I	Hours to Stage II	Hours to Stage III	Hours to Fixation	
28.3	28	3	12		24	
30.4	50	2	3		25	
44.6	44	5	11		30	
37.5	39	3		12	192	Pl. III, fig. a
40.3*	20	3			22	Pl. III, fig. a
38.9	50	2	3		9	Pl. III, fig. b
35.0	20	3	4		9	
26.0	26	3	5		6	
41.4	41		12		24	
44.4	20	3			9	
38.2	50	6	8		24	
31.4	32	11		24	72	
35.2	50	3	4	24	72	
31.0*	10	3	4	12	24	
48.9	10				192	
46.0*	46		12		24	
37.0	37		6		24	
37.5	38				168	
26.5*	26	3	4		7	
44.1	50	2½	24	45	264	
47.0*	10				192	
40.9*	20				22	
33.8	34	3	4	24	240	
38.8*	20	3		4½	9	
33.6	30	2	8		9	Plate V
47.0	20	3			22	
42.5	42	3	11		24	
41.2	50	2	2½		24	
39.8	10				240	
36.9*	36	11		24	192	
39.0*	20	3			22	

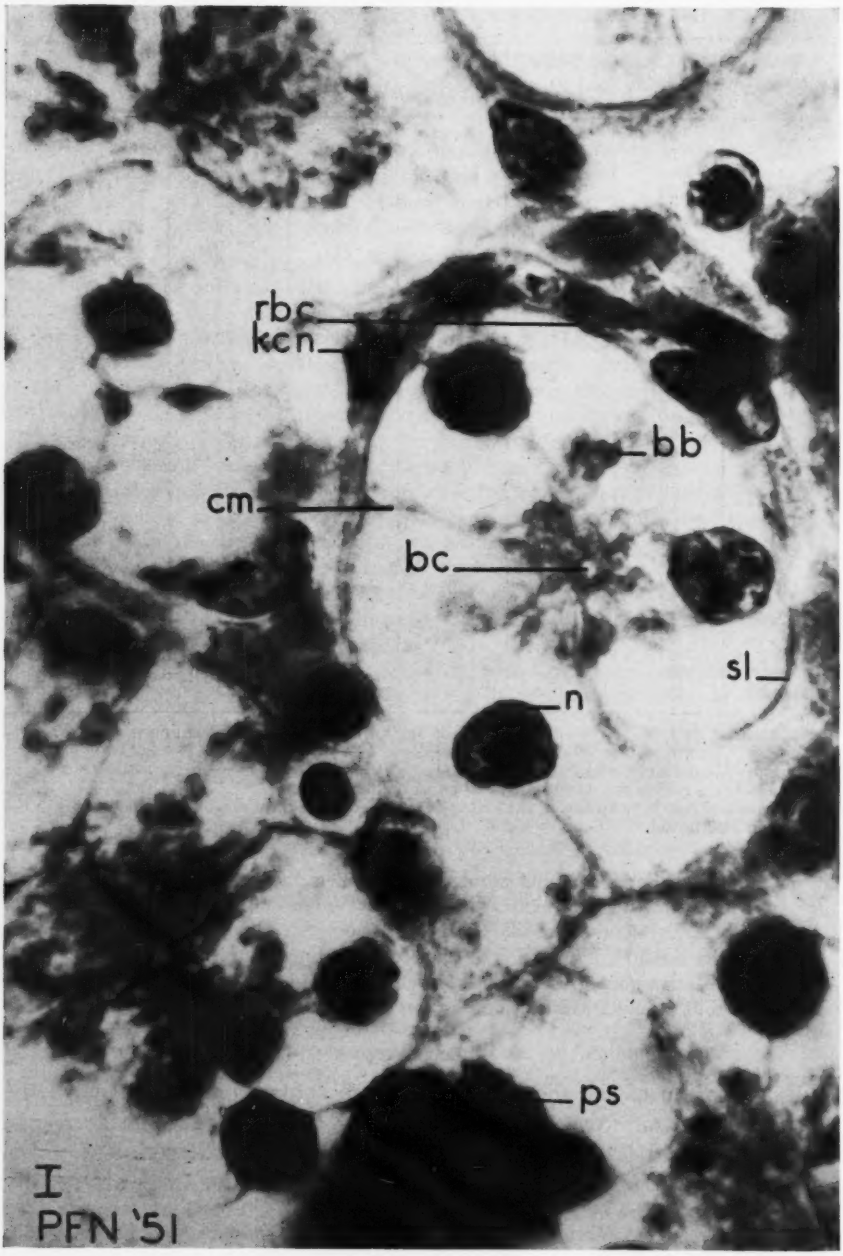
* Asterisk denotes repeated treatment of one animal. Note weight changes.
Stage I signifies loss of righting reflex.
Stage II signifies flaccid paralysis.
Stage III signifies recovery, with return of righting reflex.
Where no time is recorded in the "Stage III" space, the animal was sacrificed while in the condition last noted.

in 65° Tissumat. Serial sections were cut at 10 micra.[5] Slides were stained with iron hematoxylin, followed by a modified Masson polychrome stain. Comparable sections of the livers of experimental and control animals were stained with Gallocyanin Chrome Alum, by the method described by Lagerstedt for the identification of nucleic acid. Typical fields were photographed with oil immersion objective, on 35 mm. film. Plates were printed at a final magnification of approximately 2500×.

OBSERVATIONS

Examination of the slides prepared from control animals revealed the the prominent differences between the livers of animals sacrificed early in hibernation and the livers of control animals of this experiment. As is shown in Plate I, the bile canaliculi of the early winter frogs are readily

[5] Santomerse-S, supplied by the Monsanto Chemical Company, was used in stains and for spreading ribbons.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visible. The radially arranged cell membranes of the parenchyma are prominent. The basophil bodies are concentrated in the region of the bile canaliculus, leaving the cytoplasm near the sinusoid largely free of this material.

In the liver of the frog exposed to summer temperature through six weeks of inanition, shown in Plate II, notable differences are found. The bile canaliculi are not prominent, but are generally undetectable. The few canaliculi found in this material are of very small bore. The basophil bodies appear more widely distributed through the cytoplasm, reaching the sinusoidal border of the typical cell in heavy concentration and obscuring the radial cell membranes.

The livers of animals treated with large doses of testosterone show an opposite trend. The bile canaliculi are very large and prominent after testosterone treatment. The basophil bodies are absent or severely reduced. The cytoplasm contains elongated lacunae or vacuoles, arranged in line with the secretory axis of the cell. Between the cells are visible radial branches of the central bile canaliculi. These characteristics of the testosterone-treated liver are shown in Plate III.

The large bile canaliculi constitute a prominent feature of this material. The bile canaliculi of the control frogs appear so much smaller than those of the early winter frogs as to escape detection in almost all sections. The canaliculi of hormone-treated animals are even more prominent than those of the early winter frogs and appear several times larger.

The difference in concentration or appearance of the basophil bodies is equally striking. The cytoplasm of the cells of the control liver exhibits an over-all blue color, in low power examination, and is seen, at higher magnification, to be filled with the blue-stained basophil bodies. The over-all color of the hormone-treated material is orange; the prominent basophil bodies of the control liver are not seen. This difference in basophil body distribution and the equally prominent difference in the bile canaliculi are typical of all testosterone-treated animals.

Approximately half of the hormone-injected animals exhibited intracellular lacunae, which appeared to be arranged along the line of the secretory axis of the cell. These elongated vacuoles appeared closely applied to the nuclei of the parenchymal cells and were more prominent in the portion of the cell bordering on the sinusoid. Some were seen to extend

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PLATE 1. Masson preparation of liver fixed in February. Total magnification 2500 diameters.

Labels: bb—basophil bodies; bc—bile canaliculus; bcb—bile canaliculus branch; cm—cell membrane; kcn—Kupfer cell nucleus; l—intracellular lacuna; n—nucleus of hepatic parenchyma cell; ps—pigment spot; rbc—red blood cell in sinusoid; sl—sinusoid lining.

n
sl
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almost to the bile canaliculus. No direct, open connection to sinusoid or bile canaliculus was seen. The lacunae appeared empty, and no indication was seen of what their contents might have been before treatment with solvents in the preparation of the slides.

In the livers of some of the hormone-treated animals, there were intercellular canaliculi, radially arranged in relation to the central canaliculi. The intercellular canaliculi were seen, in some instances, to communicate directly with the central bile canaliculi. They differed from the intracellular lacunae in location; rarely appearing close to a nucleus and being most prominent in the region of the central bile canaliculus, rather than in the sinusoidal region, where intracellular vacuoles were most prominent.

Sections stained with Lagerstedt's Gallocyanin Chrome Alum for the identification of nucleic acid exhibited the structure shown in the Masson-stained material. Plate IV shows the appearance of the liver of a control frog with GCA stain. Nuclei and basophil bodies are stained strongly. The cell membranes are visible in some areas and the bile canaliculi are very fine or undetectable.

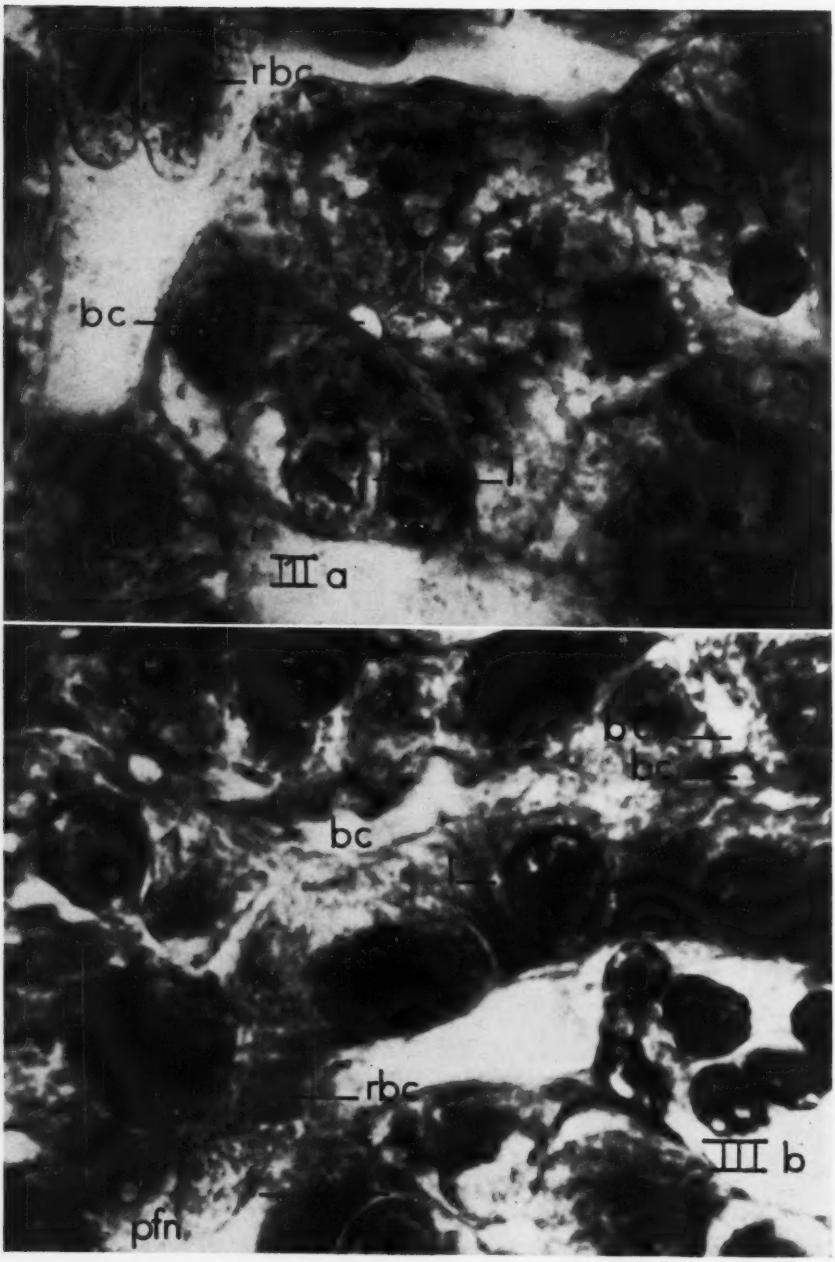
In Plate V is shown a GCA stained section of the liver of a testosterone-injected animal. No stained cytoplasmic granules are found. The central bile canaliculi are very large and are accompanied by radially-arranged intercellular canaliculi. An irregular reticulum of stained material appears to be present in some of the canaliculi.

With both Masson and GCA stain, the hormone-treated liver differs from the control liver in the much greater size of the bile canaliculi, the presence of radial intercellular canaliculi and elongated intracellular lacunae, and the diminution of absence of basophil bodies. In GCA stained material, the basophil bodies stain like the basophil cytoplasmic inclusion of the rat liver, described by Langerstedt as ribose nucleic acid granules.

The livers of animals injected with acacia without hormone exhibited the characteristics of the uninjected controls. They showed none of the features of the testosterone-treated livers. The bile canaliculi were undetectable or very fine; no intracellular lacunae or radial intercellular canaliculi were seen; and the basophil bodies were prominent.

The control treatments, with carbon tetrachloride, phosphorus, tannic acid, India Ink and Trypan Blue did not appear to influence these differences. None of the structural characteristics of hormone-treated liver was found in any animal without hormone injection. The livers of frogs in-

PLATE 2. Masson preparation of liver fixed in August after six weeks inanition at prevailing summer temperatures. This animal received no hormone. Basophil bodies fill the parenchyma cells, obscuring the cell membranes. Bile canaliculi are very fine or escape detection. 2500×.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jected with sesame oil and peanut oil, in another series of experiments, showed changes similar to those described by Mannerfelt (1947) for oil-treated rats.

DISCUSSION

The differences in biliary system and cytoplasmic basophil bodies which were observed in this investigation have not been described in other reports of the structure of liver after sex hormone treatment.[6] This divergence of result may be related to one or more of the details of the procedure employed in the present investigation. One such detail is the fact that the present investigation employed frogs, while other reports have been based upon mammals or birds, rather than amphibia. The much greater size of the amphibian cell, with consequent ease in discerning detail of structure, may be of significance. The individual doses of hormone administered in the present work were much larger than those reported for other investigations, and may have produced effects of greater magnitude or of different quality. These effects may have been rendered more readily visible by the modified Masson staining technic, which was developed for the differentiation of hepatic structures.[7]

The report of Korenchevsky (1941) describes the effect upon the basophil bodies of rats produced by castration and subsequent treatment with several steroid hormones, including testosterone. It was found that the basophil bodies, depleted after gonadectomy, were restored to normal number and size after treatment with small doses of steroid hormone, over a period of sixteen weeks. This increase in basophil bodies is the opposite of the marked diminution found in the present experiment. This difference may be related to the dose size used or to the duration of the

[6] Subsequent to the preparation of this report, Williams, W. L., C. V. Lowe and L. Thomas—Anat. Rec. 112: 402, 1952, "Effects of Cortisone Upon Hepatic Parenchyma of Rabbits," has described basophil body changes in the livers of rabbits treated with adrenal steroids.

[7] M. A. Benards, now of the University of Michigan, provided invaluable assistance.

PLATE 3. Masson preparations of livers fixed after summer inanition and hormone treatment. 2500×.

Figure a. Animal P2, pretreated with phosphorus. Body weight 40 gms. Total hormone—58 mg. in two doses; first dose 8 days before fixation, second dose 22 hrs. before fixation. Figure b. Animal TA1, pretreated with tannic acid. Body weight 35 gms. Single dose of hormone, 20 mg., 9 hrs. before fixation.

Figures show absence of basophil bodies, large size of bile canaliculi, presence of radial branches of bile canaliculi and of intracellular lacunae, close to parenchymal nuclei and lying along secretory axis of cell.

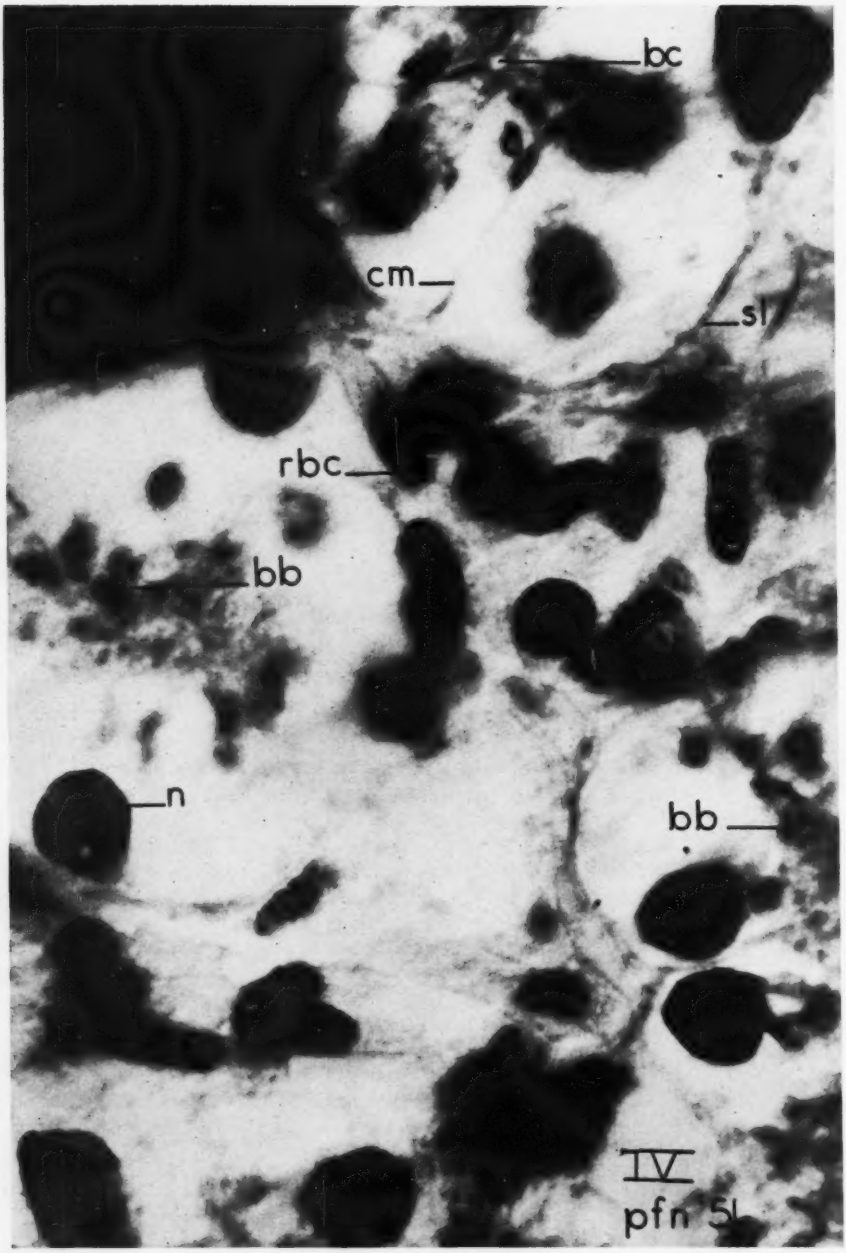


PLATE 4. Gallocyanin preparation of control animal, after summer inanition without hormone. 2500×.

hormone treatment. These factors are now being investigated, in amphibia and mammals.

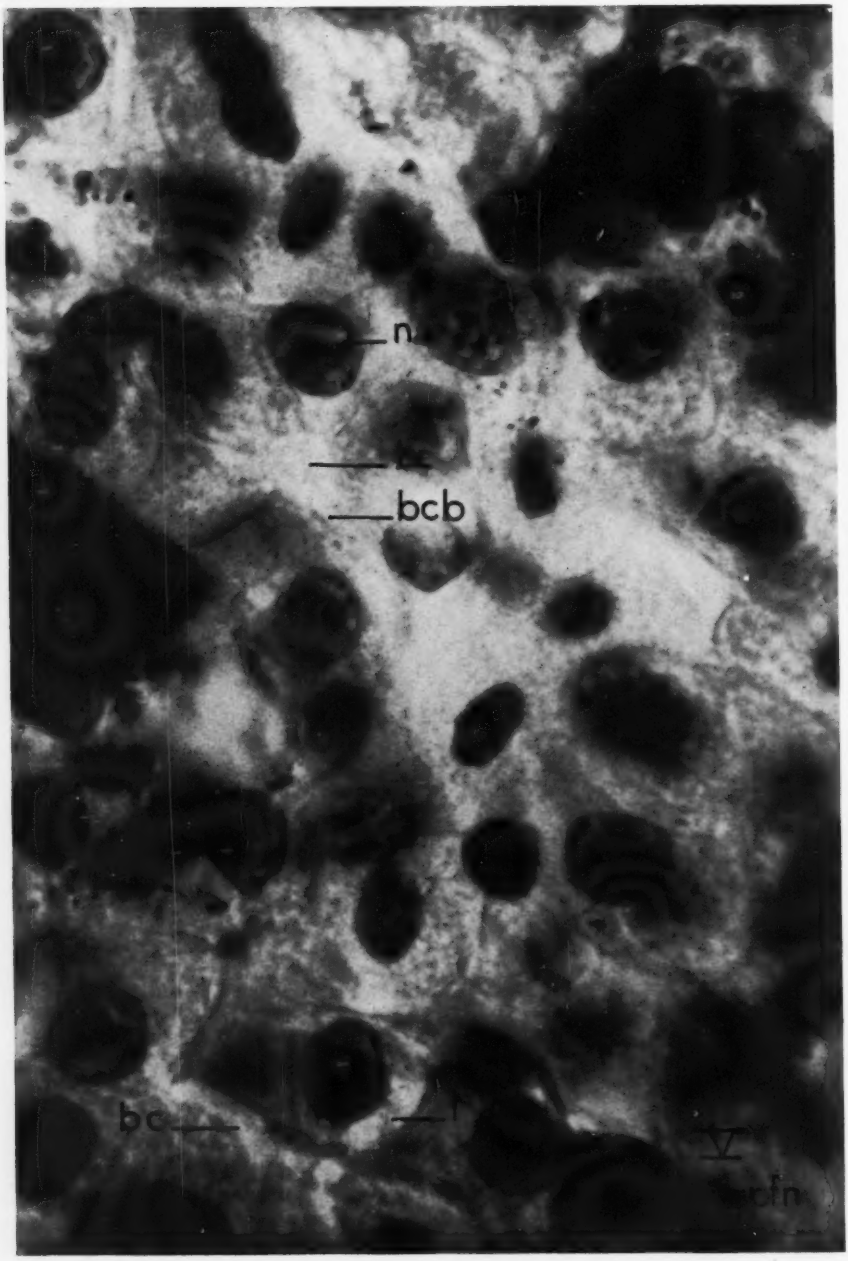
The possibility of a fundamental difference in the nucleic acid metabolism is suggested by investigations of rat liver in dietary restriction, reported by Noel (1923), Kosterlitz (1947), Lagerstedt (1949) and others. These reports describe the diminution of basophil bodies in inanition or dietary restriction. The frogs of the present investigation showed no loss of basophil bodies with inanition, but exhibited strong indications of an increase in this material. The similarity of hepatic structure and function among amphibia and mammals, described by Knisely (1948), suggests that the significance of interclass differences may be limited.

No description of the state of the basophil bodies occurs in other reports of liver structure after sex hormone treatment. Selye (1939) reported no significant change in the liver of the mouse after treatment with testosterone, although similar treatment with estrogen had been found to be followed by prominent changes leading to cirrhosis. Other reports resemble that of Selye, in this point. Blackman (1944) and associates found an increase in liver weight in testosterone-treated puppies, but observed no histological differences to account for this change. Mannerfelt (1947) detected a slight thickening of the intima of the hepatic artery in testosterone-treated rats. He reported the presence of fat vacuoles in the parenchymal cells of all oil-injected animals, with or without hormone. Similar vacuoles were found in frogs, in unpublished studies with Langan.

The tentative identification of the basophil bodies by their staining with GCA may indicate a possible relation between their depletion and the changes observed in the bile canaliculi. Ribonucleic acid has been related to secretion by Claude (1943), Stowell (1945), Davidson (1947), Caspersson (1940) and others. The appearance of the central bile canaliculi and of the radial canaliculi, described by Pollister (1932) in Amphiuma, is not inconsistent with the possibility of secretory activity.

Randavel (1948) has established a relationship between steroid hormone and ribose nucleic acid more directly. Using chemical methods, he found a 49% increase in the hepatic ribonucleic acid of the pigeon after four days of treatment with estrogen. By *in vitro* experiments, Sweat and Samuels (1949) have related nucleotide to the destruction of testosterone by liver tissue.

Indirect indication of a possible relationship between ribose nucleic acid and hepatic inactivation of steroid is provided by two series of diet experiments. Inanition or dietary restriction has been found by Noel (1923), Lagerstedt (1949) and others to result in depletion of basophil bodies. Similar treatment has been found by Biskind and Biskind (1942), Jailer (1948), Vanderlind and Westerfield (1950) and others to result in reduced ability of liver to inactivate steroid hormones.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The possible explanation of the increased size of the bile canaliculi in terms of secretory activity is consistent with another series of reports, concerning the presence of steroid hormone in the bile under physiological and experimental conditions. Pearlman and Rakoff (1949) have reported the recovery of 990 IU of estrogen from 100 cc. of the bile of a pregnant woman at term. Free estrogen was found in the bile as long as a week after delivery, in cases reported by Cantarow (1943) and associates. In experiments on dogs, Cantarow (1942) and associates recovered as much as 95% of the estrogenic activity of the substances administered, in the bile collected during the three days after the injection of estrone or alpha-estradiol.

In addition to the depletion of the basophil bodies, the large central bile canaliculi and the radial intercellular branch canaliculi of Pollister, elongated intracellular lacunae have been described as common features of the livers of testosterone-treated frogs. Similar structures have been described by Wachstein and Zak (1949), in the rabbit, and in the frog by Fraser and Fraser (1895) and by Herring and Simpson (1905, 1906). The direct communication of intracellular lacunae with sinusoids, described by Herring and by Fraser, have not been observed in this frog material.

The distinction of proper hormone effects from general injury effects was one reason for the use of the poison control treatments. It has been noted in the observations that none of the poisons produced any of the structural characteristics of the hormone-treated liver.

Evidence pertinent to the question of injury effects is provided in Table I, in which is indicated the short time required for recovery from anaesthesia, for some animals, and the short time elapsed between injection of hormone and fixation of liver, for other animals. The features of the hormone-treated liver were fully developed in as little as 6 hrs. and persisted for as long as 72 hrs. after injection and 48 hrs. after recovery.

The appearance of livers of animals treated with both hormone and Trypan Blue is particularly relevant to the possibility of cell injury. It has been shown by Williams (1950) that injured hepatic cells of mice stain intravitally with Trypan Blue in a diffuse, homogeneous coloration which later becomes granular. No staining of parenchymal cells was observed in the animals of this investigation, although Kupffer cells and pigment spots were rich in blue granules. While the earliest structural changes described by Williams began 24 hrs. after the injection of carbon tetra-

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PLATE 5. Gallocyanin preparation of hormone treated animal. 2500×.

Animal NK8, pretreated with India Ink, received one dose of 30 mg. of hormone 9 hrs. before fixation. Body weight 33 gms. Photograph shows absence of basophil bodies, large size of bile canaliculi, presence of branch canaliculi and intracellular lacunae.

chloride, the changes found in the present work were fully developed 6 hrs. after the injection of testosterone.

Evidence relevant to the question of cell injury is provided by Selye (1939), who emphasizes the freedom from necrosis and hepatitis of mice treated with testosterone, as contrasted with the frequency and fatal result of such liver injury in mice treated with estrogens.

SUMMARY

Testosterone, in anaesthetic dosages of approximately 1 mg. per gm. of body weight, was administered to female frogs by intraperitoneal injection. Responses included loss of righting reflex and flaccid anaesthesia, with depression of respiration and heart action. Livers were fixed in Bouin and Susa fluids, and stained with iron hematoxylin, Masson polychrome stain and Lagerstedt's Gallocyanin Chrome Alum stain for nucleic acid. Hormone-treated frogs showed depletion of hepatic cell basophil bodies, increased size of bile canaliculi, presence of radial branch canaliculi and elongated intracellular lacunae, when compared to control animals. GCA staining indicated the presence of ribonucleic acid in the basophil bodies of control animals and its absence from the cytoplasm of hormone-treated animals.

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THE THYROIDAL UPTAKE, RETENTION AND IODINE POOL IN RATS UNDER CONTROLLED IODIDE DIET[1]

W. A. FISH, H. CARLIN AND F. C. HICKEY, O.P.

From the Research Laboratory of Providence College and St. Joseph's Hospital, Providence, Rhode Island

INTRODUCTION

THIS laboratory has recently shown (Burns *et al.* 1951) by the in vivo method, that the biologic half-life of radioiodine in the normal human is approximately 100 days. The subjects for investigation were in the steady state and received a more than adequate iodide diet (Boston area). The divergent values given in the literature for the fractional uptake, biologic half-life, iodine pool of the thyroid and the quantity of thyroxin secreted per day suggested that a study of these factors in rats under strict dietary control might show to what extent they are dependent on the level of dietary iodide. Three levels of the steady state and two series approaching the above condition were chosen for investigation.

It was desirable to use a method which, while avoiding anesthesia and sacrifice of the animal, would allow frequent, precise and convenient measurements of I^{131} in the living thryoid. Our method is a modification of that used by Raben.[2] The apparatus used by Albert (1951), while well adapted to relative measurements, does not yield quantitative results for standardization of dose and the determination of fractional uptake by the same counter. Further, it involves the use of anesthesia and is, to that extent, less convenient for frequent measurements.

MATERIALS AND METHODS

Female rats of the Sherman strain, ranging from 180 to 220 gms. were employed. Regular photoperiods and constant temperature conditions (22–24°) were maintained. They were kept on a diet of either Purina Laboratory Chow or on a Remington diet. The former was analyzed by Tong *et al.* (1951) to contain 1.5 μg. of iodide per gm.;[3] the latter diet, 15 μg. per Kgm. (Remington, 1937). Potassium iodide supplements, when required, were administered by drinking tubes of approximately 5 ml. capacity. The tube containing 2 ml. of the iodide solution was offered to a rat which had been deprived of water for 12 hours. A thirsty adult rat can drink 3 ml. of water in less than a minute.

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[1] Supported by a research grant from the Damon Runyon Memorial Fund.

[2] Dr. M. S. Raben (1948), New England Center Hospital, Boston, Mass. Private Communication.

[3] Manufacturer's assay is 5.2 ppm. Our relative uptake results indicate this value to be rather high.

When a labeled dose was desired, 5 μc. of I^{131} was added to a 2 ml. solution containing 2 μg. of I^{127} as carrier. This method was found to be more convenient for the operator, less disturbing to the rat and just as precise as either the stomach tube or hypodermic method. One ml. of distilled water was then added as a rinse and the latter offered to the animal. For each series, an identical dose was diluted to 250 ml. and a 15 ml. aliquot was reserved as a standard in a flat bottomed, paraffin sealed vial.

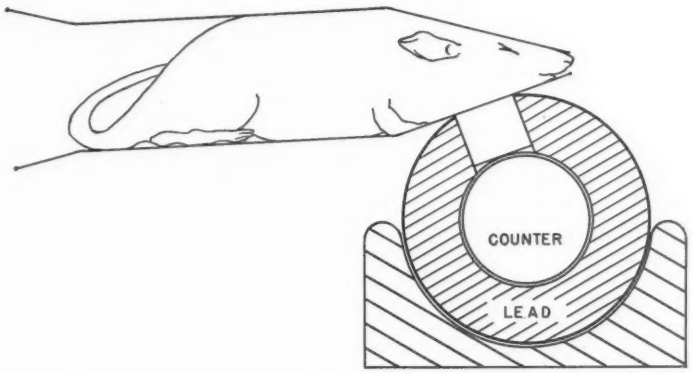

FIG. 1. Diagramatic view of counting apparatus. Shield is rotated clockwise to hold standard solution vial vertically.

Radioactivity Measurements

The counting equipment (Fig. 1) consisted of a Sylvania GG 306 gamma ray counter tube fitted into a specially designed cylindrical lead shield cased in 4 inch brass pipe. The thickness of lead was 25 mm. at all points except for the counting aperture. The latter, $1\frac{1}{4}$ inch in diameter, was bored through the shield, perpendicular to, and at the midpoint of, its long axis. Into this hole was fitted a silver plated, flat bottomed, copper cup whose lower surface was flush with the surface of the counter tube. For measuring in vivo activity, a rat was coaxed into a glass counting tube. The latter was constructed of a 9 inch length of 2 inch Pyrex tubing. A taper, three inches long and terminating in a $\frac{1}{4}$ inch hole was drawn on one end while the opposite end was flared out to approximately $2\frac{1}{2}$ inches. After being in the counting tube several times, the rats were apparently content and, in some cases, remained quiescent for periods exceeding an hour. The glass counting tube was positioned above the center of the shield cup mentioned above. The duration of the counting period varied according to the intensity of the radiation, while the frequency of counting depended on the experimental state of the animal. As an example, during the period of thyroid labeling, three successive counts, each of two minutes duration and of approximately 3000 counts per minute intensity made up one counting period. Counting periods began two hours after dose administration and were continued at two hour intervals for two days. During the retention period, two counting periods per day were always sufficient. The high efficiency of the above arrangement permitted significant counts to be obtained, in some cases, for three weeks after dosages as small as 5 μc. of radioactive iodide.

In order to determine the fraction of administered I^{131} in the thyroid gland at any time, two interrelated problems arise, a) the determination of the fraction of activity

measured in the neck region that is attributable to extrathyroidal tissues and not to the gland, and b) the ratio between the in vivo count and that of the standard solution.

Rat series were chosen which duplicated the dietary conditions of the actual experimental groups, A through E. These animals were labeled with 5 μc. of I^{131} and counted in vivo at varying intervals. These counts, C_n, measure the total activity of the gland plus that of extrathyroidal tissue exposed to the counter. The rats were then chloroformed, thyrodectomized and the neck region of the carcasses counted under identical conditions. These counts, C_c, represent the activity of the extrathyroidal tissues and general background. $C_n - C_c$, then, is the activity of the thyroid gland itself in the in vivo counting position, C_v. The extirpated thyroids were then transferred to a standard bottle containing 15 ml. of 7N potassium hydroxide solution and hydrolyzed. The bottle containing the hydrolysate was then counted by placing it in the well of the counting shield. When the hydrolysate counts, C_h, are divided by C_v, the quotient is the geometric conversion ratio, R, of the activity of the thyroid gland hydrolysate in the standard bottle to

TABLE 1. A COMPARISON OF UPTAKE AND RETENTION OF A 2μG. LABELED DOSE BY DIVISION I RATS OF THREE IODIDE LEVELS OF THE STEADY STATE

Series	Number of animals	Approximate daily oral intake of iodide in μg.	Uptake of dose in %	Time of Maximum uptake in hours	α
A	6	2.2	40.0 ± 5.7	30 ± 2	0.093 ± 0.006
	8	2.2	31.1 ± 5.8	26 ± 4	0.073 ± 0.003
B	4	0.2	54.6 ± 6.7	21 ± 1	0.105 ± 0.004
	4	0.2	49.3 ± 7.2	21 ± 1	0.101 ± 0.010
C	6	20–30	14.5 ± 4.9	12 ± 2	0.153 ± 0.009
	6	20–30	15.7 ± 2.4	Counts begun at 18 hours	0.156 ± 0.007

that of the in vivo gland. A count, C_s, is then made of the reserved standard containing 6% of the 5 μc. dose. Attention is called to the fact that the same counting apparatus is used for all counts. The fraction, F, of the dose present in the living thyroid at any given time is then calculated as follows:—

$$F = \frac{C_v}{C_s} \times R \times 0.06$$

Since F has been calculated as the fraction of the standard prepared at the same time as the dose, compensation for radioactive decay has automatically been made. For 61 rats studied, the value of R ranged from 3.4, for the smaller animals (160 gm.) to 4.0 for the larger (250 gm.), the average being 3.8 ± 0.3 for all. The value C_c, the neck background, becomes critical only when the fractional uptake is small, that is, in cases where the rats were fed 30 μg. supplements. In these cases the extrathyroidal iodine counts may be several times as great as those of the gland itself for a period of two to four hours after dosing. In all cases C_c is negligible after 48 hours.

RESULTS

Three successive experimental periods are common to each investigation: the iodine conditioning, labeling and retention periods. The experimental groups are subdivided into two divisions: Division I—held in constant iodide intake throughout the experiment, and Division II—given different iodide intakes during the various experimental periods. The animals in Division I are in iodine equilibrium, that is, the steady state resulting from constant daily intake of iodine over a sufficiently long period of time to insure that the average intake and output of the gland are equal.

Division I. The steady state

Series A. 2μg. iodide level.

Fourteen rats, divided into two groups were maintained from weaning on the Remington diet and distilled water.[4] From the 61st day to the end of the retention period (115th day) each rat was given a daily oral dose of 2 μg. of iodide as potassium iodide in distilled water. This amount, according to Levine *et al.* (1933), constitutes the daily minimum requirement to prevent goiter in the normal rat. On the 95th day the oral supplement was labeled with 5 μc. of I^{131}.

Assuming that the iodide of both the labeled dose and subsequent supplements (during retention period) are organically bound (Taurog and Chaikoff, 1947) and distributed homogeneously in a relatively short time, it is possible to calculate the quantity of iodine in the thyroid pool at any time as follows:

Let q_i = daily thyroid iodide intake
q_o = daily thyroid iodine output
Q = thyroid iodine pool
α = fractional daily loss of radioiodine from thyroid
U = the extrapolated uptake when $t = 0$.

For the steady state,

$q_i = q_o$ and Q is practically constant.

Experimentally it is found (Fig. 2) by the linearity of the plot of the logarithm of the fraction of thyroidal radio-iodine (F) retained each day, against time in days, that

(1) $F = Ue^{-\alpha t}$ and that

(2) $Q = q_i/\alpha$

This is essentially the method used by Zilversmit *et al.* (1943). Here α is the reciprocal of Zilversmit's turnover time.

[4] Since Providence City water contains only 0.004 ppm. of iodine, routine distillation was adequate.

This series, maintained on sufficient iodine for health, possessing minimum reserve and held in rigid iodine control, is considered here as a standard for comparison with other rat series. The average uptake, 35%, was reached approximately 28 hours after dosage. Maximum uptake here is considered to be the maximal amount of activity, at any time, located in the thyroid gland, although it is realized that a small portion of the activity has left the gland as labeled thyroxin prior to the time of maximum uptake. Probably the percentage error is not greater than $\alpha/2\times100$. Morton *et al.* (1942) observed that approximately 70% of the radioiodine in the plasma is thyroxin-like 12 hours after injection.

Measurements of activity of the kidney area, urine and thyroid region

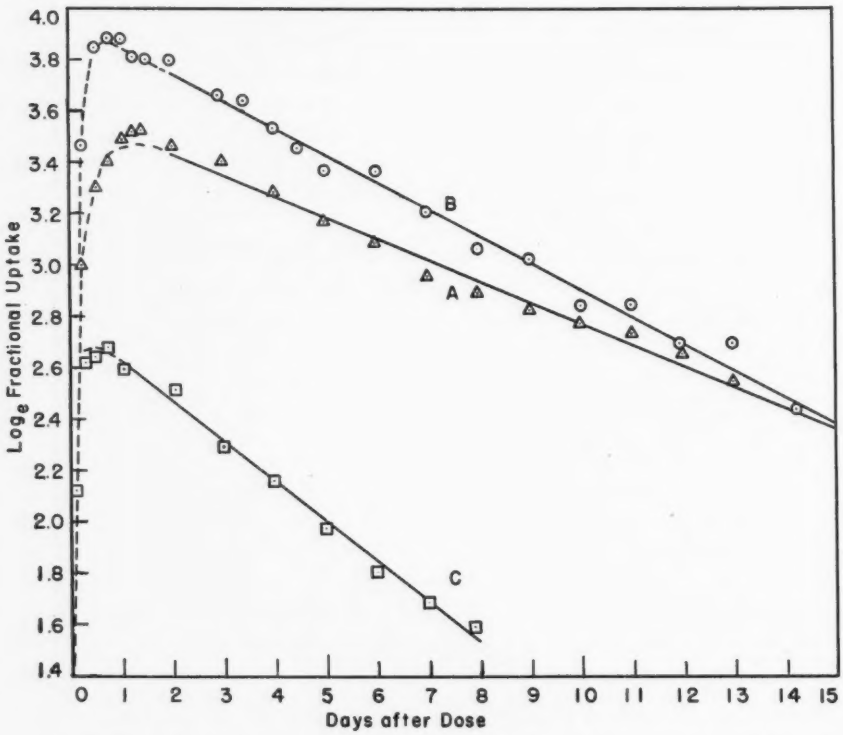

FIG. 2. Plot of natural logarithm of fractional uptake and retention against time, in days, of rats in Division I. Each point represents the average of the two groups of each series. Determinations were so frequent that many points have been omitted for clarity. Curve A—2 μg. level; B—Remington diet level; C—Purina iodide level. Solid line represents portion of curve from which α was calculated.

indicate that a negligible amount of iodine is available to the gland 24 hours after the labeled supplement is administered. Thus the product of the total amount of iodine in the labeled dose (plus that in the diet) and the per cent uptake is the daily thyroidal intake. With this series, the average value is 0.8 μg. per day. The fraction of activity retained by the gland was determined twice daily over the following three weeks. The natural logarithm of this fraction, plotted against time, led to an average value of α, that is, the slope of the resulting straight line, of 0.083, by the method of least squares. The biologic half-life was 8.3 days. The above data substituted in equation (2), led to a value of 9.6 μg. of total iodine in the thyroid pool. The calculated thyroxin secretion was 1.2 μg. per day. The possibility of reabsorption of iodide from "degraded" hormone makes the above values subject to an undetermined but definitely small error (Gross and Leblond, 1950).

Series B. Remington diet level

Eight rats, divided into two groups, were maintained from weaning on a Remington diet throughout the experiment, except for the single labeled dose containing 2 μg. of carrier iodide administrated after 60 days on the diet. This amount, although absolutely small, is however approximately 10 times that of the thyroid iodine pool. Even though this amount temporarily upsets equilibrium and reduces the value for fractional uptake, it is the only reliable one that can be employed for accurate uptake comparisons with other series of Division I.

As noted in Table 1, the average uptake, a minimal value, is considerably higher than that of series A, as would be expected. Thus, q_i, calculated with the above restrictions has a minimal value of approximately 0.1 μg.; the biologic half-life averages 6.7 days and the calculated gland pool, one day after labeling, is approximately 2.0 μg. This latter value is the sum of the pool before labeling and 52% of the labeled 2.0 μg. iodide administered.

During the first days of retention, the amount of iodine lost by the thyroid per day, is greater than the quantity of iodine available to the gland from its diet. Calculations show, for practical purposes, that approximate equilibrium will again be established in about 10 days, although a significant variation in α cannot be detected. It is realized, however, that the biolologic half-life, as a measure of thyroxin output, is not as accurate as desired for this series as a steady state, because of the *relatively* large amount of carrier used in the labeling dose. It is to be noted that this excess above equilibrium is not immediately delivered to the body, even though the gland is in a goitrous condition.

Series C. Purina diet level.

Twelve animals, divided into two equal groups, were maintained from weaning on Purina Laboratory Chow throughout the entire investigation. This represents an average oral intake of approximately 20–30 μg. iodide. When these rats reached the weight of 180 gms. they were given a 2.0 μg. labeled dose of potassium iodide.

The average value for maximum uptake, 15%, is considerably lower than that of the previous series, as anticipated. Assuming that the uptake of the 2.0 μg. dose is a fair indicator of the thyroidal uptake of the daily dietary intake, then the value of q_i falls between 3 and 4.5 μg., and Q between 20–30 μg. The biologic half-life, 4.5 days, would not be measureably affected by reabsorption of released hormone iodide since, at a maximum, less than 6% of this released iodide could possibly be taken up by the thyroid. The rate of thyroxin secretion is between 4.6 and 6.9 μg. per day.

TABLE 2. A COMPARISON OF UPTAKE AND RETENTION OF A 2μG. LABELED DOSE BY DIVISION II RATS CONDITIONED ON 0.2μG. DIET AT TWO LEVELS OF ALTERED IODIDE INTAKE DURING RETENTION PERIOD

Series	Number of animals	Approximate daily iodide intake in μg. during retention period	% Uptake of dose	Time of maximum uptake in hours	α
D	6	2.0	42.7 ± 4.6	22 ± 2	0.060 ± 0.002
F	7	30.0	48.5 ± 9.5	23 ± 2	0.078 ± 0.006 5th to 18th day

Division II. Altered dietary intake

Series D. Approaching the 2.0 μg. level from the Remington diet.

The six rats of this series were maintained from weaning to the weight of 170 gms. on a Purina diet. They were then placed on a Remington diet for 17 days prior to labeling with a 2.0 μg. dose. This interval was chosen because histological preparations of thyroid tissue of rats deprived of iodine for 17 days show the first unmistakable signs of hypertrophy. During the retention period they were given a daily 2.0 μg. supplement. The value for maximum uptake lies almost midway between the uptake values for series A and B. However, the half-life, 11.6 days, was higher than that of both the above groups, indicating a slower rate of thyroxin output. Obviously, during retention, q_o will be smaller than q_i, and it cannot be calculated unless we assume a reasonable value for Q. Because of the beginning hypertrophy and the high uptake, Q lies between 1 and 5 μg., probably closer to the lower limit. The measured value of q_i is 0.9 μg.

at the day of labeling and decreases to approximately to 0.8 μg. as the equilibrium of series A is approached. Thus, for practical purposes, q_i is substantially constant throughout the retention period.

A rapid loss of labeled thyroxin from the gland was anticipated during the first few days of retention. However, as shown in Figure 3, α remained constant throughout the period. The small value of α as compared to that of series A, indicates that Q attains its equilibrium value more rapidly than does q_o. This conclusion parallels that of Taurog and Chaikoff (1949) who have shown that in hyperthyroid rats, the ratio of thyroxin to diiodotyrosine, normally the greater fraction of organic iodine, is increased over that in the normal gland.

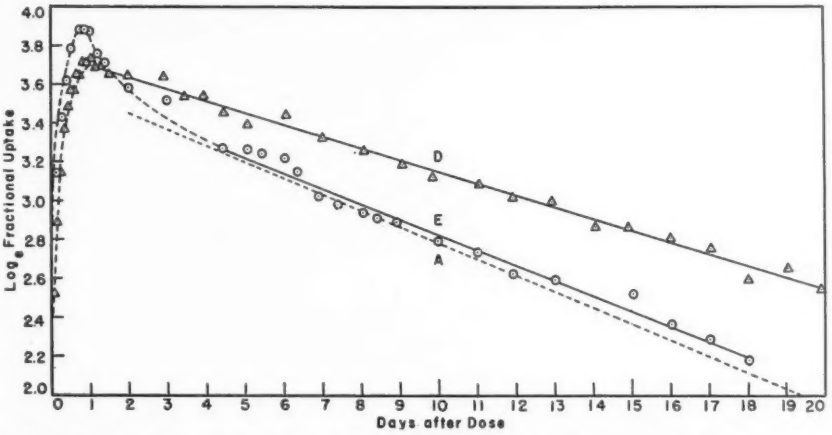

FIG. 3. Plot of natural logarithm of fractional uptake and retention against time, in days, of rats of Division II. α is calculated from portion of curve represented by solid line. The slope of Series A (broken line) is included for comparison.

Series E. Approaching the 30.0 μg. level from the Remington diet.

This series was conditioned identically to the above up to and including the labeling period. From the first day of and throughout the retention period they were given a daily 30.0 μg. supplement.

The high uptake desired for a prolonged retention study is impossible to attain with a labeled 30.0 μg. dose because of the low fractional uptake. On the other hand, since Q and q_o both depend on a measured value for q_i, it is necessary to obtain this latter value by the fractional uptake method. Auxiliary experiments were conducted on three groups of rats which were conditioned identically to series D with the following exceptions. For one group of 30.0 μg. supplement was labeled on the 1st, for the second group on the 5th and for the third group on the 8th day of the 30.0

μg. regimen. The corresponding uptakes were 11 ± 2, 11 ± 1 and $7 \pm 1\%$ respectively, yielding an average weighted value, during retention, for q_i of 2.5 μg. per day. Since extrathyroidal activity accounts for a large percentage of the total in vivo counts at the time of maximum uptake, these values are subject to 10–15% error as explained above.

As is shown in Figure 3, the rats in this series showed a more rapid loss of activity during the first third of the retention period than did the previous series. During the remainder of the period, however, the rate of loss was exponential, as in other series. However, the percentage loss per day was considerably less than that of the auxiliary groups and also less than that of series C. The iodide level of the latter corresponds, roughly, to that of this series.[5] Again, this slower rate of loss compared to that of the equilibrium level, can be explained in a manner similar to that offered for the preceding series D.

However, the initial more rapid loss, not evident in any other series, represents the "washing out" effect of activity due to an addition of unlabeled iodine equal to or perhaps greater than the existing thyroidal pool.

DISCUSSION

Investigations involving the use of I^{131} for estimating quantitative values of certain thyroid functions in the rat have yielded inconsistent results. The following group comparisons are made only on the basis of dietary iodide. It is not intended here to neglect the fact that some of the differences between our results and those of other workers may have their origin from other variables, such as pre-dosage conditioning, age, sex and strain of rats employed. However, it seems certain that the variation in values of maximum uptake and retention are not due entirely to these factors but chiefly to the failure to attain the steady state and to less precise methods of measurements.

Results comparable to Series A. In Wolff's (1951) experiments, using the in vivo technique on rats maintained on a basal diet of 0.28 μg. iodide per gm., the maximum uptake after 40 hours ranged from 12.6 to 41.2% of the dose and the half-life was 3.3 days. He obtained approximately the same half-life in animals supplemented with 10 μg. of iodide injected daily. Perry (1951), whose rats were on a 0.5 μg. iodide per gm. diet, observed a maximum uptake of approximately 22% and a 2 day half-life. Other uptake values, under similar conditions of diet, ranged from 7.6 to 58% (Taurog *et al.* 1947; Leblond and Gross, 1948; Gross and Leblond, 1951).

[5] Rats on a 30 μg. iodide supplement for 15 days have a maximum uptake of 15%, the same as that of the Purina series.

Dougherty *et al.* (1951) report that rats on a diet supplying 1–2 μg. iodide per 100 gms. of body weight, averaged less than a 0.5 μg. thyroidal iodine pool; on a similar diet, 0.3 μg. iodide per gm. of food, Wolff and Chaikoff (1948) report an average pool of 9.3 μg., a value almost identical to that of our Series A.

Results comparable to Series B. Albert's (1951) in vivo studies of animals on a Remington diet yield a half-life of approximately 2.0 days, a thyroidal iodine pool of 6 μg. and a thyroxin secretion rate of 2.5 μg. per day. The last two values seem high in view of the fact of the diet regimen. The control rats (40–50 gms.) of Randall *et al.* (1951) maintained again on a Remington diet, have a half-life of approximately 8 days, in close agreement with our series B. Randall and Albert (1951), with Remington fed rats estimate biologic decay to be 0.36% per hour (6% per day). The uptake for the control rats of Money *et al.* (1951) averages 53%; the value for *Q* as reported by Levine *et al.* (1933) averages approximately 0.8 μg. per 100 gms. of animal. Both of these values agree closely with those of our rats.

Results comparable to Series C. Taurog and Chaikoff (1947) with rats on a diet probably similar to Purina Chow as indicated by their 20% uptake, calculate an average rate of thyroxin secretion to be 2 μg. per 100 gms. body weight per 24 hours. Taurog *et al.* (1950) analyzed the thyroid gland chemically and found an average value for *Q* to be approximately 17 μg. Both of the above values are in general agreement with our Purina series.

SUMMARY

An in vivo technique, allowing convenient and quantitative measurement of thyroidal I^{131} in the rat, without anesthesia, is presented. The uptake and retention of iodine are studied at three levels of the steady state and two levels approaching the steady state.

In agreement with previous workers, the degree of uptake is inversely related to the amount of iodine available to the gland. At the 2μg. daily intake level, the maximum uptake is 35%, corresponding to 0.8 μg. iodide, the biologic half-life, 8.3 days, the thyroidal iodine pool, 9.6 μg., and thyroxin output, approximately 1.2 μg. per day. For the Remington dietary state, the corresponding values are 52%, or approximately 0.1 μg. uptake, 6.7 day half-life, and approximately a 1.0 μg. pool. An accurate calculation of hormone output could not be made because of the relatively large (2.0 μg.) carrier used in labeling. At the Purina dietary level, the uptake is 15%, representing 3.0–4.5 μg. iodide, a half-life of 4.5 days, an iodine pool of 20–30 μg., and a secretion rate of 4.6–6.9 μg. thyroxin per day. The above values compare favorably with those reported by certain other workers using different techniques.

The two groups of rats, interrupted from the Remington dietary level by daily supplements of either 2.0 or 30.0 μg. iodide, when compared to their respective steady state series, show a loss of thyroidal I^{131} at a slightly but significantly slower rate. Apparently this indicates that the glandular iodine pool attains its equilibrium value more rapidly than does that of the hormone output.

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THE EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE INJECTION ON GLYCOGEN STORAGE IN SKELETAL MUSCLE[1]

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IN A previous report (Leonard, 1952), it was shown that testosterone propionate exhibited marked glycopexic and glycostatic effects on the striated muscles of the perineal complex of male rats. It cannot be assumed that the results and conclusions obtained from the study of these particular muscles would necessarily be applicable to skeletal muscles in general, particularly since this subject is highly controversial (Leonard, 1952, for literature review). This report is concerned with the effect of androgen on the glycogen levels of those skeletal muscles which would be considered more representative of the body musculature of the rat, i.e., the *rectus femoris* of the thigh and the abdominal muscles. In addition, glycogen determinations were made on the *cremaster* muscle which is sensitive, morphologically, to androgens, similar to the peroneal muscles.

METHODS

Adult male and female rats of the Long-Evans strain were selected for uniformity of age and body weight within each experiment. The operative procedures and post-operative care were the same as previously described (Leonard, 1952). Testosterone propionate[2] in peanut oil was injected; the controls received peanut oil only. The control animals were investigated simultaneously with the treated animals in each experiment.

During the 24 hour period immediately preceding autopsy, the rats were deprived of all food and were placed on wire-bottom cages to prevent coprophagy. Water was available at all times. The rats were anesthetized by an intraperitoneal injection of Nembutal (6 mg. per 100 grams body weight) to the point of surgical anesthesia before obtaining the muscles.

The muscles taken for analysis were removed from each rat in the following order: rectus femoris, abdominal muscles, and cremaster. It had been observed previously that very low glycogen levels obtained in the rectus femoris if this muscle was removed after the perineal muscles in the same rat. When the above procedure was followed, the glycogen levels were not lowered in the remaining muscles sufficiently to require the use of separate rats for each muscle. However, it seemed advisable to limit the number of muscles to be studied from any one animal.

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[1] This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

[2] The testosterone propionate used in this study was furnished through the generosity of the Schering Corporation, Bloomfield, New Jersey.

To remove the rectus femoris, the rat is placed on its back and the skin is separated from the anterior surface of the thigh. The entire muscle mass from the anterior surface of the thigh is removed by scissors with little or no loss of blood from the rat. The rectus femoris can be recognized by its distinctive fascial sheath and is easily separated from the surrounding muscles and connective tissue. The muscle will contract only infrequently before it is placed in the KOH solution. To remove a sample of the abdominal muscles, the skin over the abdomen is displaced to one side, an incision is made along the *linea alba* and a rectangular block of muscle (600–800 mg.) is taken. To remove the cremaster muscle, the scrotum is cut in the mid-line and one of the cremaster muscles is gently dissected as free as possible from the surrounding connective tissue before it is severed from the body in the inguinal region. Any remaining connective tissue is removed from both inside and outside of this muscular sac as rapidly as possible before weighing.

All muscles were weighed on a torsion balance before placing them in tubes of 30% KOH. The tubes were placed in a boiling water bath immediately. The method of separation of the glycogen was essentially that described by Good, Kramer and Somogyi (1933). After recovery of the glycogen by centrifugation, it was dissolved in a volume of water equal to the original volume of KOH (2–3 ml.) and reprecipitated with alcohol (1.5 volumes). The glycogen was recovered again, by centrifugation, dissolved in a known volume of water and aliquots taken so that the amount of glycogen determined ranged from 50–125 gamma of glucose equivalents.

The anthrone method for determining glycogen was employed (Morris, 1948; Viles and Silverman, 1949; Seifter *et al.*, 1950), using a Klett-Summerson colorimeter with a red filter. The anthrone reagent was made up 18–24 hours before using each day and the reaction was carried out according to the recommendations of Seifter *et al.* (1950). The glycogen in the muscles is reported as mg. of glucose per 100 grams of muscle, wet weight.

EXPERIMENTAL

Previous studies indicated that the maximum decrease in glycogen levels of the perineal muscles of rats was reached 7 days after castration

TABLE 1. THE EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE ON GLYCOGEN LEVELS OF SKELETAL MUSCLES

Experiment	Glycogen (mg. per 100 gr. muscle)*					
	Thigh m.		Abdominal m.		Cremaster m.	
	Exper.	Control	Exper.	Control	Exper.	Control
Male, castr., 7 days	373 ±16†	412‡ ±14	484 ±28	535‡ ±51	470 ±20	590 ±31
Male, castr., 32 days	385 ±10	457 ±11	463 ±11	538 ±19	465 ±18	575 ±15
Male, castr., 3 days+6 mg. testos. p. in 6 days	520 ±26	441 ±17	605 ±19	489 ±19	620 ±16	483 ±22
Female, spay., +5 mg. testos. p. in 5 days	558 ±12	401 ±18	640 ±29	430 ±16		
Hypsect. male, +3 mg. testos. p. in 3 days	358 ±14	250 ±11	392 ±18	271 ±16	520 ±22	400 ±18

* The averages were based on the results from 10–16 rats in each instance.
† Standard error of the mean.
‡ Difference between the means of the experimental and control animals not statistically significant. All other differences are statistically significant with P values not greater than .02.

(Leonard, 1952). Consequently, glycogen determinations in the rectus femoris, abdominal and cremaster muscles were made following a similar period of castration. The results in Table 1 show that castration effected a decrease in the glycogen concentration in all 3 muscles but only in the cremaster muscle was this difference statistically significant. When male rats were castrated and the muscles were removed for analysis 32 days later it was observed that a significant decrease in the glycogen level occurred in all 3 muscles (Table 1). The percentage decrease in glycogen in the cremaster muscle, in this experiment, was not different from that which occurred 7 days after castration.

To determine if testosterone propionate would elevate muscle glycogen, a group of male rats was castrated and 3 days later 1 mg. of the hormone was administered daily for 6 days to each animal. The castrated controls received oil. The results in Table 1 indicate that a statistically significant increase in glycogen was obtained in these 3 muscles of the rats, treated with androgen, when compared with the respective controls.

A similar experiment was conducted on female rats, ovariectomized for periods of 20–50 days. Spayed females were used to circumvent any possible effects of ovarian secretions. Testosterone propionate injected at the level of 1 mg. per day for 5 days significantly increased the glycogen content of the thigh and abdominal muscles, and on the basis of percentage increase, these muscles of the females appeared to be more responsive than those of the castrated males (Table 1).

Finally, experiments were performed to determine if testosterone propionate exhibited a glycostatic effect on these 3 muscles in hypophysectomized male rats according to the methods devised for testing glycostatic effects of growth hormone (Russell and Wilhelmi, 1950). Male rats were hypophysectomized and 6 days later were injected with 1 mg. of the male hormone per day for 3 days. The glycogen levels of the rectus femoris, the abdominal and cremaster muscles were found to be markedly higher in the treated rats than in the controls (Table 1). Thus testosterone propionate enabled the muscles of the fasted hypophysectomized rat to retain glycogen (glycostatic action).

DISCUSSION

When considering the effect of castration on the glycogen content of skeletal muscle, it is necessary to take into account the particular muscle and the length of time the animals have been castrated. The cremaster muscle, like those of the perineal complex, is quite sensitive, morphologically, to male hormone. In rats castrated for 32 days this muscle appeared to be $\frac{1}{3}$-$\frac{1}{2}$ the size of that in the normal controls. Similar atrophic changes occur in this muscle after castration in the ground squirrel

(Wells, 1937). Thus castration for a period of 7 days was sufficient to lower the glycogen content in the cremaster but did not do so in either the thigh or abdominal muscles to a significant extent. However, castration for 32 days was sufficient to lower the glycogen content of all 3 muscles significantly.

Injection of testosterone propionate raised the glycogen levels in these muscles in both castrated male and ovariectomized female rats. If the thigh and abdominal muscles can be considered as representative of the skeletal muscles of the body, in contrast to the male hormone "sensitive" cremaster and perineals, it may be concluded that testosterone can exert a glycopexic action on skeletal muscles in general. Although the experiments on the castrated male and female rats were not exactly comparable, it did appear as though the muscles of the female were more responsive to testosterone than were those of the male. Schuman (1940) reported that testosterone increased the glycogen level in the quadriceps muscle of the castrated rabbit and more recently Larizza and Notario (1951) claimed similar results in the guinea pig. The conclusions of the latter investigators were based on very few animals and the data from the experimental and control animals overlapped widely.

A glycostatic effect of testosterone propionate was demonstrated on all 3 muscles which were examined in the hypophysectomized males. No attempt was made to determine if it was possible to maintain the glycogen level equal to that found in the castrated rats. Many quantitative aspects of this problem remain to be investigated. There seems to be no doubt that the muscles of the hypophysectomized rat are very responsive to the effects of testosterone on glycogen storage.

It seems that the increased glycogen storage in muscle tissue induced by injections of testosterone is but another manifestation of the anabolic property of this steroid. Many investigators have reported on the protein anabolic properties of testosterone and allied steroids (Kochakian, 1946). In the latter instance measurements are made on the ability of the animal to excrete less nitrogen in the urine when male hormone is administered. This is assumed to express the protein anabolic activity of the hormone. It is also well known that an increase in the amount of carbohydrate ingested by an animal will likewise cause a retention of nitrogen as measured by a decrease in the amount of this substance excreted in the urine (Munro, 1951). Furthermore, this effect of extra carbohydrate appears to act independently of functional islets of Langerhans and of the pituitary (Bancroft, Geiger, and Hagerty, 1951). Therefore it is suggestive that the effect of testosterone in maintaining and increasing glycogen levels in skeletal muscle may be a part of the mechanism by which the hormone exerts its influence on nitrogen retention and protein anabolism in the animal.

SUMMARY

Adult male rats which were castrated for a period of 7 days showed a statistically significant decrease in the level of glycogen in the cremaster muscle but not in the thigh (rectus femoris) or abdominal muscles. If the period of castration was extended to 32 days a significant decrease in glycogen occurred in all 3 muscles. Injections of testosterone propionate into castrated male rats increased the glycogen content of these skeletal muscles above that of the castrated controls. Testosterone propionate increased the glycogen content of the thigh and abdominal muscles of spayed female rats. It was possible to demonstrate a glycostatic effect of testosterone propionate in these 3 muscles, in hypophysectomized male rats. It is concluded that the male hormone can influence the glycogen level of skeletal muscles when observed in fasting male and female rats.

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THE DIABETOGENIC AND GROWTH-PROMOTING ACTIVITIES OF GROWTH HORMONE (SOMATOTROPIN) IN THE DEVELOPING CHICK EMBRYO

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THE experiments reported here were designed to determine whether or not growth hormone normally produced by adult mammals influences embryonal growth. Fetal gigantism occurring in human diabetic or potentially diabetic mothers (Krist and Futcher, 1948) has been suggested as due to excessive maternal growth hormone production. Several investigators have demonstrated a diabetogenic effect of growth hormone in intact (Cotes et al., 1949; Campbell et al., 1950; and Milman and De Moor, 1950) as well as partially depancreatectomized adult mammals (Milman and Russell, 1949, and Houssay and Anderson, 1949). However, such a response could not be elicited in growing puppies and kittens (Young, 1941, 1944, 1949), or in pregnant or lactating cats and dogs (Young, 1938, 1946).

Evans, Simpson and Li (1948) have reported that growth hormone produces a continuous growth in normal adult female rats. Recently, Reid (1952) has shown that the ratio of diabetogenic to growth-promoting activity is approximately a constant, thus suggesting that the two activities may be attributed to the same structure in the same protein molecule. Nevertheless, there is some doubt that the two effects are to be ascribed to a single substance (Raben and Westermeyer, 1952).

In general, investigators studying the effects of growth hormone have controlled food consumption, but have not mentioned control of water intake (Brody, 1945). In this regard the chick embryo is a particularly suitable animal for testing the effects of growth hormone, since food consumption is limited to that available in the yolk sac and water intake to the passage of controlled atmospheric moisture through the shell and membranes.

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MATERIALS AND METHODS

A preparation of anterior pituitary growth hormone was supplied by Armour and Co.* (Lot No. K-40805R) and was dissolved in sterile saline adjusted to pH 8 with dilute NaOH. New Hampshire eggs, purchased from a local hatchery, were used throughout the experiment, which was carried out during November and December. The eggs were incubated at a temperature between 99.5 and 99.75° F., with moisture read at 86–87° F. on a wet bulb thermometer.

Chorio-allantoic injections were started on the thirteenth day of incubation. Previous trials at earlier stages resulted in an extremely high mortality rate, leaving few eggs for study. The eggs were divided into two control and four experimental groups of 30 eggs in each group. One set of controls was sham operated and another received physiological saline adjusted to pH 8 in the same manner as the growth hormone preparation. Among the experimental groups four different doses, in total amount of 5, 10, 20 and 40 μg. of growth hormone were administered; this was carried out either as a single injection on the thirteenth incubation day, or over a five-day period with one injection every other day, starting with the thirteenth incubation day.

Blood samples of 0.1 ml. were taken from newly hatched chicks by cardiac puncture and the blood sugar determined by the method of Somogyi (1952). All chemical determinations and body weight measurements were carried out within twelve hours after hatching; body weights were determined with a tortion balance (sensitivity 0.1 gm.)

TABLE 1. EFFECT OF GROWTH HORMONE ON THE BLOOD SUGAR AND BODY WEIGHT OF NEWLY HATCHED CHICKS INJECTED DURING EMBRYONIC DEVELOPMENT

Sex	Group	Number of chicks	Blood sugar		Body weight	
			Mg. %*	Range	Grams*	Range
Male	Sham operated	13	163.7 ± 4.6	139.2–191.4	44.5 ± 0.8	39.9–48.2
	Saline controls	9	164.4 ± 6.7	120.3–196.2	41.9 ± .08	38.7–46.0
	Growth hormone					
	5 μg.	11	159.8 ± 9.3	135.0–215.7	44.3 ± 1.0	38.0–48.0
	10 μg.	15	168.8 ± 5.8	141.3–213.6	45.4 ± 0.8	40.2–51.6
	20 μg.	14	169.2 ± 7.2	122.4–215.7	45.9 ± 0.9	41.5–52.3
	40 μg.	8	206.3 ± 4.9	184.2–215.7	47.6 ± 1.5	42.5–55.3
Female	Sham operated	9	162.4 ± 8.2	143.3–191.4	42.8 ± 1.6	33.6–47.9
	Saline controls	6	166.9 ± 8.4	139.2–196.2	42.5 ± 1.1	38.6–46.1
	Growth hormone					
	5 μg.	10	184.5 ± 10.5	143.4–211.5	45.1 ± 1.2	38.3–50.5
	10 μg.	9	177.3 ± 9.3	162.3–211.5	45.8 ± 0.9	41.6–49.5
	20 μg.	7	182.9 ± 6.6	162.3–207.3	44.7 ± 1.0	40.9–47.7
	40 μg.	8	182.9 ± 6.7	162.3–207.3	46.8 ± 1.4	41.7–54.6

* Standard deviation of the mean.

RESULTS AND DISCUSSION

From the data shown in Table 1 it is apparent that, in general, both the blood sugar level and the body weight are higher in newly hatched chicks receiving three injections of growth hormone during embryonal life than in sham operated or saline injected controls. Furthermore, those experimental groups which showed the most marked response in weight increase,

* We are indebted to Dr. Irby Bunding of Armour and Co. for the supply of growth hormone preparation, Lot #K-40805R.

also showed the most marked elevations in blood sugar. Thus, in males a significant difference in body weight and blood glucose level was observed only in the group receiving 40 μg of growth hormone, while in females significant differences were observed at all four dosage levels. However, even in males, as indicated by the upper limit of the range of variation, individual experimental animals showed responses greater than controls, although the average body weight and blood glucose level was not significantly higher than controls. Furthermore, there was no direct correlation between dosage and degree of response, since in females 5 μg. elicited as great a response in body weight and blood sugar increase as that obtained with greater quantities of growth hormone.

In general, the sex difference is rather striking, although males receiving 40 μg. of growth hormone show a higher average growth as well as diabetogenic response than females receiving this dose level. Thus while 5 μg. of growth hormone is capable of eliciting a significant response in female embryos, male embryos require a much greater quantity of hormone.

In contrast, a single injection on the thirteenth incubation day of the same quantities of growth hormone failed to elicit a significant diabetogenic or growth response in embryos of either sex; this observation indicates that 13-day chick embryos are probably not sufficiently mature to respond to growth hormone, and that even the initial injection of the series of three was probably without effect.

Observations on hatchability showed that while in sham operated controls hatchability was 77 per cent, in the saline control group it was only 53 per cent; this inhibition was probably due to the alkalinity of the saline. Among the experimental groups, only those chicks which received 10 μg. of growth hormone showed an increased hatchability rate (80 per cent), while it was 70 per cent for both the 5 and 20 μg. levels, and only 53 per cent for the 40 μg. groups. Thus even in experimental groups the pH of the injection solvent may be a factor; at the 10 μg. dose level there is probably sufficient hormone to produce an increased hatchability rate while alkalinity is sufficiently low to permit the effect to become manifest. Larger doses of hormone probably also inhibit hatchability.

From the foregoing data it may be concluded that growth hormone (somatotropin) can produce a growth-promoting and diabetogenic response in the developing chick embryo, which can be detected immediately following hatching. Brody (1945) has pointed out that about 90 per cent of the weight gain attributed to growth hormone in the investigations of Lee and coworkers (1934, 1935, 1936) may have been water gain. The controlled humidity in the present experiments makes such a factor unlikely, but a future report will clarify this point.

Particularly noteworthy is the striking sex difference as manifested by the greater sensitivity of the female chick embryo to growth hormone. This

observation is in agreement with observations by Evans et al. (1931, 1938) in adult rats, but is contrary to that observed in adult C3H mice bearing a transplantable mammary adenocarcinoma; in the latter experiments male mice showed a greater response to somatotropin than females (Smith *et al.*, 1952).

SUMMARY AND CONCLUSIONS

Anterior pituitary growth hormone (somatotropin) exerts a diabetogenic and growth-promoting action when administered during embryonal developing of the chick embryo, starting with the thirteenth incubation day. Female embryos show considerably greater sensitivity to this hormonal stimulus than male embryos. There appears to be no direct correlation between the quantity of hormone injected and the degree of response, although the most marked average response occurred in male embryos receiving 40 μg. of growth hormone, the largest dose used.

Growth hormone may also affect hatchability rate; maximal hatchability was obtained with 10 μg. of hormone. The alkalinity of the hormone medium may play a role in diminishing hatchability rate, but large doses of growth hormone may also inhibit hatchability.

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DECREASED EXCRETION OF CORTISONE METABOLITES IMMEDIATELY FOLLOWING ASCORBIC ACID TREATMENT

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IN THE course of studying the role of ascorbic acid in adrenal cortex function it was observed that the vitamin is capable of prolonging the hematologic actions of exogenous and of endogenous cortical hormones (Bacchus, Altszuler, Heiffer, 1952a, b). The data were interpreted as indicating that the ascorbic acid in some manner may be responsible for either a diminished excretion or a diminished inactivation of these hormones. At any rate, detectable amounts of the hormones persisted in the animals which received simultaneous ascorbic acid treatment.

The present paper reports data which indicate that the excretion of 17-ketosteroids in the first 24-hour urine sample following cortisone is diminished by ascorbic acid treatment. This diminished excretion of the metabolites corresponds very well with the observed period of prolongation of hematologic effects noted above.

MATERIALS AND METHODS

Female adrenalectomized Wistar rats were the animals of choice in this experiment. It is generally accepted that the chief source of 17-ketosteroids in the female is the adrenal cortex (Heard, 1948; Selye, 1952). In the adrenalectomized female, however, the ovary may produce small amounts of 17-ketosteroids (Dorfman, 1948). The animals were bilaterally adrenalectomized under ether anesthesia at least three weeks prior to experimentation and were maintained on an ad libitum fare of 0.9% NaCl and Purina Laboratory Chow throughout the period of study.

Group I rats received single intraperitoneal injections of 100 mg. ascorbic acid (Na ascorbate, Roche) per 100 grams body weight. Fifteen minutes later the animals were given single subcutaneous injections of 2 mg. cortisone acetate (Merck) per 100 grams body weight. Group II rats received saline injections (instead of ascorbic acid) and the same dosage of cortisone as above. Group III rats received single intraperitoneal injections of ascorbic acid at the same dosage as above, while Group IV received saline solution only. Immediately after the injections the animals were placed in individual metabolism cages and supplied with food and fluid. A urine sample of the first 24 hours was collected from each animal. The 17-ketosteroids in the urine were determined by the method of Drekter, *et al.*, (1952) in which dehydroisoandrosterone was used as standard.

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The results are expressed as 17-ketosteroids as mg. of dehydroisoandrosterone per the 24-hour urine sample.

RESULTS

The data in Table I indicate that the 17-ketosteroid excretion in adrenalectomized female rats which received no cortisone is relatively low. Ascorbic acid did not affect this pattern. The excretion of 17-ketosteroids in the animals which received saline plus cortisone is significantly higher ($P = <0.05$) than that in the rats which were treated with ascorbic acid and

TABLE 1. EXCRETION OF 17-KETOSTEROIDS IN THE 24-HR. URINE SAMPLE FOLLOWING INJECTION INTO FEMALE ADRENALECTOMIZED RATS OF SALINE, ASCORBIC ACID, SALINE PLUS CORTISONE, OR ASCORBIC ACID PLUS CORTISONE

Group	No. rats	Treatment	17-KS as mg. dehydroisoandrosterone in first 24-hr. urine sample ±S.E.*
I	8	Ascorbic acid plus cortisone	0.128 ±0.015
II	8	Saline plus cortisone	0.500 ±0.157
III	4	Ascorbic acid	0.075 ±0.011**
IV	3	Isotonic saline	

* S.E. $= \sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$

** Values were grouped together since they were similar.

cortisone. The data may be interpreted to suggest that ascorbic acid depresses the excretion of 17-ketosteroids following cortisone treatment.

DISCUSSION

It is probable that most of the 17-ketosteroids observed in these urines were derived from the injected cortisone. This is in consonance with generally accepted facts (Sprague, Power, Mason, Albert, Mathieson, Hench, Kendall, Slocumb, and Polley, 1950; Selye, 1952). The diminished excretion suggests that the level of cortisone is maintained in the blood in animals which received ascorbic acid along with cortisone. This finding may explain the prolongation to 24 hours of the hematologic (eosinopenia, lymphopenia, and polymorphonuclear leukocytosis) actions of cortisone in animals with such treatment (Bacchus, Altszuler, and Heiffer, 1952 a, b). It may be noted that the hematologic actions of adrenocorticotrophin injections into non-adrenalectomized rats is also prolonged to 24 hours by simultaneous ascorbic acid treatment (Bacchus, Altszuler, and Heiffer, 1952 b). Preliminary data indicate that a similar pattern as above may be present in the excretion of 17-ketosteroids in such animals.

The above findings may provide a reasonable explanation for previous observations to the effect that non-specific stressors (e. g. epinephrin,

trauma, cold) fail to activate the pituitary-adrenal axis in ascorbic acid treated intact rats, mice and guinea pigs (Bacchus et al., 1951; Bacchus, Heiffer and Altszuler, 1952; Bacchus and Altszuler, 1952). Recent work by Eisenstein and Shank (1952) confirms the observation made by Bacchus et al., (1951) that ascorbic acid pretreatment prevents the depletion of adrenal ascorbic acid and adrenal cholesterol following epinephrin. All of the above data are the basis of a tentative hypothesis that ascorbic acid maintains the level of cortical hormones in the blood (for a longer time, hence their manifestations) and in this manner increases the threshold for pituitary stimulation. The mechanism proposed by Sayers (1947) involves a regulatory cycle whereby a high level of cortical hormones in the blood depresses pituitary-adrenotrophic function, whereas a low titer stimulates pituitary-adrenotrophic function. The suggested mechanism of action of ascorbic acid described above is in consonance with this theory of Sayers (1947).

(The observation that ascorbic acid excess diminishes the excretion of cortisone metabolites (17-ketosteroids) prompts reevaluation of the data of Clayton and Prunty (1951) and of Nadel and Schneider (1951) who reported increased excretion of cortical hormone metabolites in scurvy. Those authors interpreted their data as indicating adrenal hyperactivity in scurvy. Such interpretation is in disagreement with the findings of McKee, et al., (1949) who reported low liver glycogen values in such animals, and of Treager, et al., (1950) who reported normal responses to ACTH in scurvy patients. It is suggested that the increased 17-ketosteroids excretion observed by those authors in scurvy may be due, not to adrenal hyperactivity, but rather to the absence of ascorbic acid, which maintains the levels of circulating hormones. Studies are being conducted on the mechanism of action of ascorbic acid in this respect.)

SUMMARY

The action of ascorbic acid on the excretion of the 17-ketosteroid metabolites of injected cortisone was investigated in adrenalectomized female rats. The data indicate that ascorbic acid significantly diminishes the excretion of 17-ketosteroids in the first 24-hour urine sample following the treatments. Since it was observed that similar vitamin treatment prolongs the action of such doses of cortisone for 24 hours, the present findings are interpreted as indicating that the vitamin serves to maintain the levels of circulating cortisone.

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ACTION OF LIVER ON THYROID HORMONE FOLLOWING INTRASPLENIC IMPLANTATION OF THE THYROID

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THE intravenous injection of a large dose of thyroxine is followed by increased biliary excretion of iodine (Kendall, 1919), and by the excretion of thyroxine in the feces (Gross and LeBlond, 1947, Monroe and Turner, 1948). However, only a small per cent of the injected thyroxine is excreted in the bile (Gross and LeBlond, 1947, Monroe and Turner, 1948). For one hour after thyroxine is injected intravenously the hormone is excreted in the bile unchanged. Thereafter the injected thyroxine is eliminated in the bile as an unknown compound (Taurog, Briggs and Chaikoff, 1951). This unknown compound is hydrolyzed during its passage through the gut, and releases free thyroxine which is not absorbed (Taurog, Briggs and Chaikoff, 1952). When physiological doses of thyroxine are injected, the percentage fixed by the liver and excreted in the feces is even smaller (LeBlond, 1949).

It has been shown previously that transplanted thyroid tissue functions in the same manner as does the thyroid in its normal position as gauged by uptake of iodine (Marine, 1916) and by response to thyrotrophic hormone (Marine and Rosen, 1934). In view of these observations it was thought that transplantation of the thyroid into the spleen should offer a means of studying the effect of the liver on thyroid hormone.

Should the liver remove appreciable amounts of thyroid hormone from the circulation in the presence of normal levels of circulating hormone, transplantation of the thyroid into the spleen should be followed by manifestation of thyroid hormone deficiency, and perhaps by structural changes of the implant.

METHODS

Male and female rats originated from Wistar Strain and bred by the Albino Farms, Redbank, N. J., were used. At the age of six weeks the entire thyroid gland was removed from the neck and transplanted into the spleen in five male and five female animals. Equal numbers of control animals were sham operated at the same time. The control and the operated animals were observed for one year after transplantation. In six animals one lobe of the gland was placed in the spleen, the other was left in situ. Six animals whose only thyroid tissue was in the spleen were given 0.05% thiouracil in the drinking water for fourteen days prior to autopsy, which was performed one year after the trans-

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TABLE 1

	Males		Females	
	Operated	Controls	Operated	Controls
Initial wt.	110	102	75	81
Final Wt.	460	448	261	272

Weight gain of operated (thyroidectomized, one thyroid lobe implanted in spleen) and control rats during one year.

plantation. Ten rats who had all their thyroid tissue in the spleen were autopsied at various times after the transplantation. At the time of autopsy the animals were carefully observed for the presence of adhesions. The region of the splenic implant was fixed in Bouin Solution, and stained with Hematoxylin-Eosin. All animals were allowed free access to food (Purina dog chow).

RESULTS

The animals whose only thyroid tissue was in the spleen reached the same weight at the end of one year as did the controls (Table 1).

The structure of the gland was not altered by its intrasplenic position

FIG. 1. Section of spleen with thyroid implant 1 year after implantation (×130)

Fig. 1 (1 year after transplantation). The transplanted gland underwent hyperplasia in animals given thiouracil (Fig. 2).

The presence of functioning thyroid tissue in the neck did not alter the survival or the structure of the transplanted thyroid. The structure of the transplant in animals who had one lobe of the thyroid in the neck was identical with that of animals whose only thyroid tissue was in the spleen. Results were identical in male and female rats.

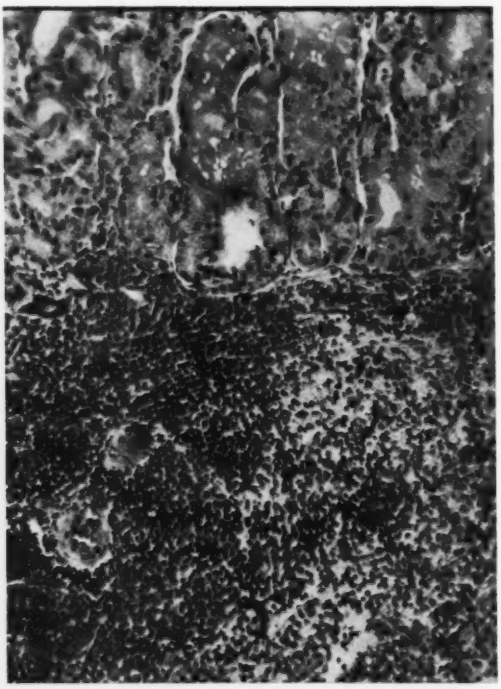

FIG. 2. Section of spleen with thyroid implant 1 year after implantation. Animal received thiouracil for 14 days prior to autopsy (×130).

DISCUSSION

While this paper was in preparation, Bondy (1951) reported similar experiments. Our findings as well as those of Bondy, show that the thyroid implanted into the spleen not only remains viable, but also functional, as indicated in our experiments, by the normal growth rates, and in Bondy's experiment by the uptake of I^{131}. Bondy studied the transplants for 110 days, whereas our observations were extended up to one year.

Transplantation of the thyroid into the spleen causes the secretion to

pass entirely into the portal circulation, provided no adhesions are formed which would permit channeling of some of the hormone directly into the systemic circulation. Such adhesions were absent in our experiments. If the liver would "inactivate" all or the major part of the thyroid hormone delivered through the portal circulation, whether by degradation of thyroxine or by biliary excretion, one should expect (a) manifestation of hypothyroidism, or (b) hyperplasia of the thyroid implant through increased TSH secretion from the uninhibited pituitary gland. The normal growth curve, indistinguishable from that of the sham operated controls, indicates that adequate amounts of thyroid hormone pass through the liver and reach the systemic circulation. These amounts of hormone are also adequate to regulate pituitary TSH secretion and to prevent excessive secretion of the latter. This is evidenced by the absence of hyperplasia of the splenic implant. Alternatively, the absence of thyroid hyperplasia in the splenic implant might have been due to an irresponsiveness of TSH of the thyroid tissue in an abnormal environment. However, such an explanation is untenable in view of the fact that administration of thiouracil causes hyperplasia of the thyroid located in the spleen.

These observations indicate that physiological amounts of thyroid hormone are not "inactivated" by the liver; or if some "inactivation" takes place, it is of such minor degree, that amounts adequate to maintain euthyroidism (gauged by growth and maintainance of normal TSH secretion) pass the liver into the major circulation. On the other hand, the fact that iodide and thyroxine are excreted in the bile following administration of large doses of thryoid hormone, suggests that under such conditions the liver does inactivate some thyroid hormone either by destruction or by biliary excretion. The recent isolation of triiodothyronine from the blood of humans (Gross and Pitt-Rivers, 1952) may alter the concept that iodide in the bile results from the hepatic inactivation of the thyroid hormone. It is possible that the liver may alter thyroxine to form triiodothyronine. The iodide might be excreted in the bile without any decrease in circulating thyroid active material. However, the presence of free thyroxine in the bile following the intravenous injection of thyroxine is evidence that the liver does remove thyroxine from the circulation.

It furthermore becomes apparent that the role of the liver in metabolizing and/or excreting thyroxine differs, at least quantitatively, in some important points from that in handling estrogens. Following transplantation of the ovary into the spleen, the uterus and vagina exhibit the picture of estrogen deficiency, and the pituitary gonadotropin secretion is uninhibited, causing growth and tumor formation in the intrasplenic ovary (Biskind and Biskind, 1944). Only minute amounts of estrogen, inadequate

to maintain the uterine and vaginal mucosa or to suppress pituitary FSH secretion, escape the action of the liver (Bernstorf, 1951).

Another interesting aspect is the observation that intrasplenic implants of the thyroid appear to "take" and grow just as readily in animals in which one lobe was left in situ as they do in animals in which all the thyroid tissue was removed from the neck. This is in contradistinction to the experience with other glands, notably the adrenal cortex, transplants of which are reported to take only in animals with no other functioning cortical tissue (Ingle and Higgins, 1938). The significance of this different behavior is not apparent and warrants further study.

SUMMARY

The growth of animals whose thyroids are transplanted to the spleen is normal. The transplanted thyroids have normal structure, and will become hyperplastic if the thyroid hormone production is blocked by thiouracil. These observations indicate that thyroid hormone sufficient to support growth and to regulate the rate of pituitary thyrotropin production escapes "inactivation" (by destruction or biliary excretion) when the hormone enters the portal circulation.

The author gratefully acknowledges the continued interest of Dr. K. E. Paschkis in this investigation.

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THYROIDAL METABOLISM OF IODINE IN THE SHARK *SCYLIORHINUS* (SCYLLIUM) *CANICULA*

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THE physiological role of the thyroid gland in the fishes remains largely enigmatic, since most research has demonstrated a lack of response of oxygen consumption to thyroidal hormone (Root and Etkin, 1937; Hasler and Meyer, 1942; Smith and Everett, 1943) in this group of animals. It is possible that the piscine thyroid is involved in salt metabolism and osmoregulation (Koch and Heuts, 1942; Fontaine and Koch, 1950), and there is some suggestion that thyroxine may play a role in certain specialized phases of maturation or "metamorphosis" in fishes (Sklower, 1928; Harms, 1934). Indeed, it has been established that the pigmentary changes, for example, in transformation of the young "parr" trout to the "silvery smolt" form are influenced by thyroxine treatment (Landgrebe 1941; Robertson, 1949). On the other hand, Stokes (1939) has shown that thyroxine will not initiate metamorphic changes in the larval lamprey. Using thyroidal material derived from the Parrot fish, Smith and Matthews (1948) were able to demonstrate some stimulation of oxygen consumption in another fish, the white grunt, *Bathystoma*.

Such data logically raise the question of whether or not the thyroids of fishes produce the same hormone as do the glands of higher vertebrates. In this connection it is known that thyroidal material from the lamprey can provoke metamorphosis of frog tadpoles (Sembrat, 1927; Horton, 1934) in the manner typical for thyroxine. From chemical and radioautographic work it seems well established that thyroids of fishes concentrate radioiodine in organic form (Gorbman and Creaser, 1942; Wolff and Chaikoff, 1947; Matthews and Smith, 1948; Leloup, 1951). Finally, by fractionation of butyl alcohol extracts of shark thyroids of different species, Wolff and Chaikoff (1947) and Leloup (1951) have tentatively established that at least one form of such organically bound radioiodine is probably thyroxine.

Because of the fragmentary and dispersed nature of the data on iodine fixation and hormonal synthesis by the thyroid gland of fishes we elected to examine more exhaustively the question of iodine metabolism in a single

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selachian species, the "petite roussette" of the French Atlantic coast, *Scyliorhinus* (*Scyllium*) *canicula*. Using the technique of radio-chromatography it has been possible not only to identify thyroxine in the thyroid, but also the precursor iodinated amino acids. Second, the rate of thyroxine synthesis was determined by sampling at various intervals after administration of radioiodine and, finally, the microscopic distribution of the radioiodine was observed by radioautography.

METHOD

The forty *Scyliorhinus canicula* used in these experiments were caught in nets in the immediate vicinity of the Laboratoire Maritime of the Collège de France, Concarneau,

TABLE 1. TOTAL UPTAKE OF IODINE BY THYROID GLANDS OF *S. canicula*

Time interval	Sex	Length cm.	Body Wgt. gms.	Thyroid mg.	Uptake percent
(Hours)					
1	♀	46.5	275	16	.045
1	♀	49.0	353	16	.011
1	♂	42.7	235	11	.080
6	♂	38.2	153	9	5.0
6	♀	45.2	207	17	20.5
6	♀	56.5	560	59	34.3
6	♀	46.5	275	17	2.73
6	♂	49.5	325	12	3.40
17	♂	40.7	178	5	2.6
17	♂	50.2	350	24.5	11.4
23	♀	46.0	260	12.0	.62
23	♂	49.5	315	12.5	.49
24	♀	46.0	260	8.0	.16
24	♀	45.3	240	13.0	.093
24	♀	35.0	122	4.0	6.20
24	♀	41.3	220	8.0	.11
24	♀	31.8	125	3.0	4.92
30	♀	39.4	170	6.0	—
30	♀	46.0	305	20.5	1.24
30	♂	39.7	178	8.5	—
30	♂	60.5	610	25.5	1.40
30	♂	54.0	465	30.0	8.73
41	♂	59.5	600	4.0	0.85
41	♂	43.5	205	2.5	9.70
41	♂	43.5	200	2.5	3.65
96	♂	46.2	250	8.0	.17
96	♀	40.8	182	5.0	.25
96	♀	42.8	260	15.0	.45
96	♀	43.3	225	9.5	.42
96	♂	57.5	520	29.0	.25
96	♀	66.0	870	99.5	1.67
144	♀	67.0	1045	191.0	.21
144	♀	46.0	235	11.0	.13
144	♀	38.0	141	6.5	.18

Finistère, France. They were used within one to three days following capture, being kept at all times afterwards in large concrete basins through which a flow of fresh sea water was maintained. The longest period of such detention was six days, and no feeding was attempted during this time. The fishes were of varying size and of both sexes, and their distribution in the experiment is detailed in Table I. Five of the forty animals were not injected with I^{131}, and their thyroids served as histological controls.

Radioactive iodine, I^{131}, as KI, free of carrier iodide was injected intraperitoneally in doses of 20 to 50 microcuries (dosages calibrated against a Tracerlab "simulated radioiodine reference source"). At intervals of 1, 6, 17, 24, 30, 41, 96 and 144 hours after injection groups (Table I) of animals were sacrificed. At these times the thyroids were dissected out and weighed. One weighed fraction of each thyroid gland was "wet ashed" in 5% KOH, one was fixed in Bouin's fluid, and the largest part was frozen for chemical analysis. Aliquots of the ashed thyroid tissue were measured for gross radioactivity to determine the gross percentage of the originally injected dose of I^{131} concentrated by the thyroid (calculations corrected for physical decay). The Bouin-fixed tissue was prepared for radioautography by applying the histological sections against no-screen X-ray film squares. The sections were afterwards stained for histological study and comparison with the radioautographs. The frozen tissue from all animals of the same post-injection interval was pooled and was crudely extracted overnight at 10° C. with normal saline solution after having been sliced as finely as possible with sharp razor blades. The filtered crude extract was then hydrolyzed according to previously described techniques of tryptic digestion, and the hydrolysate was extracted with butyl alcohol for quantitative one-dimensional filter paper chromatography (Roche *et al.*, 1950a, 1950b), using butanol-acetic acid or butanol-ammonia as solvents. Known monoiodotyrosine, diiodotyrosine, and thyroxine were used for purposes of identification in the chromatography, either in parallel with the hydrolysates or as carriers for them.

RESULTS

Uptake of administered I^{131}. As the data in Table I demonstrate, the accumulation of radioiodine by the thyroid of *Scyliorhinus* is rapid, but also, in comparing individuals, it is extremely irregular. Using the ratio of the weight of the thyroid to the body weight as a rough index, even as little as one hour after injection there is already a significant accumulation (0.01 to 0.08% of the administered dose is in the thyroid; thryoid = .005% to .006% of body weight). The highest values obtained were at the six-hour post-injection interval (20.5% and 34.3%). Though the values are quite irregular, several observations of a general nature may be made. A peak of tracer iodine uptake occurs between six and twenty hours after injection, and after that there is a rapid loss of radioiodine from the thyroid. This conclusion seems justified by the fact that there not only are peak values at this time, but also because there are no low values for I^{131} content, as there are 23 hours or more after injection. Between one and six days after injection, though the content of the radioiodine is lowered, it still represents a very significant concentration if the thyroid-body weight ratio is used as a rough index.

Radiochromatography. Because of insufficient material in certain of the

thyroid samples, or insufficient radioactivity, or both, it was possible to prepare satisfactory radiochromatographic analyses only for the post-injection intervals of 6, 17, 24, 41, and 96 hours. Nevertheless, the results seem to give a clear picture of the general character and rate of hormonal synthesis (Figure 1).

At six hours after injection it may be seen that 70 per cent of the tracer iodine already is in the organic form of iodinated tyrosine, monoiodotyrosine being slightly in excess. Despite the high level of I^{131}-accumulation, only a relatively small fraction of the iodine is present as radioiodide. At

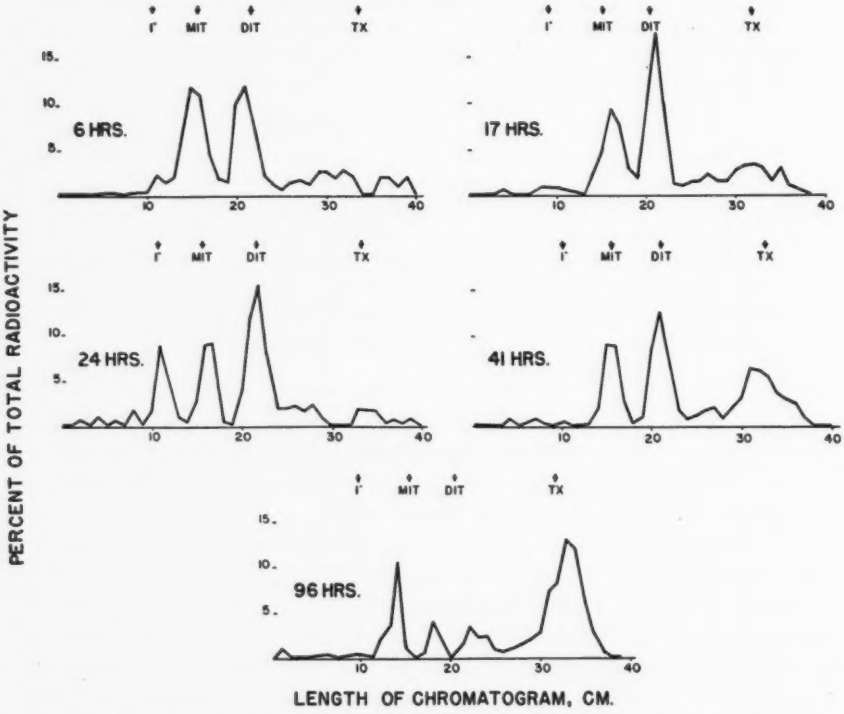


FIG. 1. Filter paper chromatography of tryptic hydrolysates of thyroid tissue removed at different times after injection of I^{131}. The figures show the distribution of radioactivity due to I^{131} along the length of the chromatogram. The probable chemical form of the I^{131} is indicated at the top of each figure. Each arrow indicates the position of a known chemical compound chromatogrammed in parallel with the thyroidal hydrolysate. Abbreviations: I, iodide; MIT, monoiodotyrosine; DIT, diiodotyrosine; Tx, thyroxine. It should be noted that the percentages indicated are percentages of the total radioactivity of the entire chromatogram. There may be, therefore, considerable variation in absolute radioactivity from one time interval to the other.

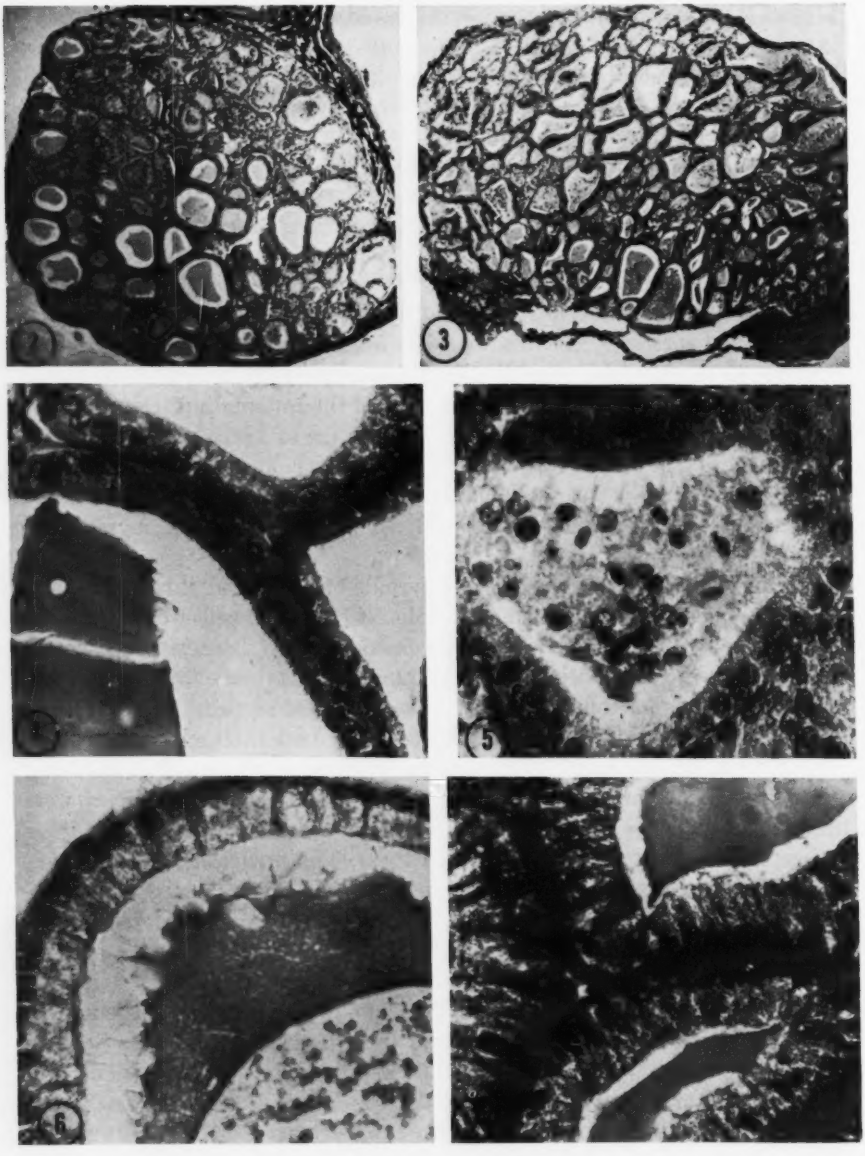
17 and 24 hours diiodotyrosine is increased at the expense of monoiodotyrosine, and the first small proportion of thyroxine (18.5%) is found in the 17-hour group. The absence of thyroxine in the 24-hour group is difficult to explain but will be discussed later. At 41 hours the thyroxine fraction is further increased at the expense of the iodinated tyrosines, and at 96 hours, when the gross radioactivity level of the whole thyroid is much reduced, 53% of the remaining I^{131} is in the form of thyroxine. No appreciable amounts of inorganic iodine were found after 24 hours.

Histology and radioautography

Untreated control animals. In the thyroids of untreated Roussettes a considerable histological variation was found, and it was therefore important to characterize this "normal" variation in order to evaluate the changes which might be considered effects of the radioiodine accumulation. The features which were found to have a range of variation in untreated animals were, (1) the height of the follicular epithelium, (2) size of the follicles, (3) presence of intracolloidal cells (i.e., cells in the follicular lumen), (4) structure of the colloid, (5) localized pycnotic changes in follicular cells.

The most usual follicular cells in thyroids of untreated Roussettes were cuboidal in shape, but in the smallest follicles columnar cells were ordinarily found (Figures 2, 4). Follicles varied considerably in diameter, apparently according to the size of the animal. Young thyroids were fairly uniformly composed of smaller follicles (about 40 cells in cross section). In thyroids of large adults, especially females, follicles were two to three times larger, and often extremely large follicles were found, as much as five or six times the diameter of the smaller follicles. Some of the largest follicles were bizarre in shape, being adapted to the contours of neighboring follicles, rather than having the usual rounded form. The nuclei of epithelial cells normally were basal, or almost basal in position; the chromatin was granular, and there was a single nucleolus. The cytoplasm was characteristically full of uniform red granules or globules easily visible without use of oil immersion lenses (i.e., with "high, dry" objective), (Figure 4). Pycnotic degenerative changes in isolated follicles, or in part of the epithelium of certain follicles, were frequently found in untreated animals. In cells so affected the nuclei were shrunken and irregular in shape, and their chromatin was more or less clumped. The red cytoplasmic granules of such cells tended to be fewer, larger, and more irregular in size.

A feature which usually was correlated with the relative degree of epithelial pycnosis was the presence of cells in the colloid of certain follicles. All thyroids, however, had a few follicles containing such intracolloidal cells (Figure 2). The derivation of most of these cells seemed to be, clearly,



Figures 2–7, Histologic Sections:

FIG. 2. Low magnification of thyroid gland of untreated female shark. Note that some follicles contain intracolloidal cells.

FIG. 3. Low magnification of thyroid gland of shark killed 41 hours after 35 micro-

from the epithelial cells, since usually, despite the pycnotic nucleus, the characteristic cytoplasmic granules were obvious and relatively unchanged (Figure 5). Some of the intracolloidal cells, however, seemed definitely to be of the macrophage type. The colloid itself in untreated animals was quite uniform, being as a rule dense, eosinophilic, and little, if at all, vacuolated at its edge. A few mitotic figures could always be found.

I^{131}-treated animals. After radioiodine injection in the sharks there was superimposed upon this normal pattern of variation a series of morphological changes. However, these changes were not by any means as severe as those seen in mice, for example, after higher dosages of radioactive iodine (Gorbman, 1950). Although a few mitotic figures were found in the 1-hour post-injection group, very few, if any, were found after that time. The pycnotic areas were found more frequently in injected animals, the most extensive pycnotic changes being in the 6 to 24-hour post-injection group. Corresponding increases in occurrence of intracolloidal cells were noted in this time interval (Figure 3). In the 4-day and 6-day post-injection series no more than normal pycnosis was observable. An epithelial change not seen in uninjected animals was the presence of follicles composed of extremely tall columnar cells in the 4-day and 6-day groups (Figure 7). A frequently observed change in the colloid of treated animals was a tendency to assume a granular or globular (Figure 6) form. Associated with the latter type was the development of one or more central colloid-free areas. This is in contrast to the usual "vacuoles" which develop at the cellular border of the colloid. Some of the large globules of colloid were basophilic rather than eosinophilic in staining affinity.

The radioautographs of the 1-hour post-injection groups were extremely weak or entirely negative. All other thyroids of I^{131}-injected Roussettes yielded satisfactory radioautographs. These, without exception, showed the radioiodine to be located in the colloid.

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curies of I^{131}. Note increased number of intracolloidal cells.

FIG. 4. High magnification of thyroidal epithelium of untreated shark. Note density of colloid, number of eosinophilic granules in cytoplasm.

FIG. 5. Intracolloidal cells in thyroid of shark killed 41 hours after 35 microcuries of I^{131}. Some of the cells are very similar to the bordering epithelium and contain similar granules. Others are in different stages of pycnosis.

FIG. 6. Thyroidal epithelium of shark killed 72 hours after 50 microcuries of I^{131}. The cells are swollen and tall. Nuclei are irregular in shape. Cytoplasmic granules are dispersed. The colloid is of two types: a granular ring-shaped type near the cells, and a central type consisting of globules of different sizes. The granular ring yields a radioautograph, but not the globular region.

FIG. 7. Thyroid of shark killed 41 hours after 35 microcuries of I^{131}. Cells are abnormally tall, as in Fig. 6, but cytoplasmic granules are not dispersed, and colloid is normal.

DISCUSSION

Iodine accumulation rate. It would appear from the data summarized in Table 1 that iodine newly entered into the body fluids of the Roussette is quickly taken up by the thyroid, and that soon it is quickly released into the system from the thyroid. The maximum accumulations of 20% and 34% observed only six hours after administration of the tracer compare favorably with maxima observed in similar studies of mammalian thyroids and thyroids of salmon (La Roche, 1950), but the retention of the metabolized iodine (i.e., its "biological half-life") is apparently shorter. It may be noted as a factor of possible significance that the shark lives in a relatively iodine-rich environment (about 60 micrograms iodine per liter of sea water) and the need for iodine conservation is perhaps not as great as in fresh water or terrestrial organisms. Because of the continuous supply of iodine from the environment, it would seem that a rapid loss and failure to store metabolized iodine could well be tolerated by the organism. In this connection, Leloup (1951) observes that whereas the iodine content of the blood serum of sharks is higher than that of mammals, the iodine content of the thyroid is considerably less. In a small series of four *S. canicula* sacrificed between 2.5 and 16.5 hours after injection of radioiodine, Leloup found no thyroidal uptake of I^{131} greater than .01%. The thyroid of one of two similarly treated *Scyllium stellare* had 0.45% of the dose of I^{131} 72 hours after injection. It may be that a larger series of animals, given the variation we have observed, might show more agreement between our results and those of Leloup. In one interesting detail there is good agreement between the figures of Leloup and the present data. As long as 72 hours (Leloup) or 144 hours (present series) after administration of the I^{131}, the thyroid contains an important (even if reduced) concentration of the isotope.

It is of interest at this point to note the results obtained by La Roche (1950) in the Atlantic salmon. He found that whereas most of the thyroidal radioiodine uptake in this teleost species occurred within the first 24 hours, additional accumulation took place, at a slower rate, until 70 hours after administration of the isotope. The peak thyroidal uptake of I^{131} varied from 5% to 20%, but this could be reduced to less than 2% if 10 to 60 parts per million of stable iodine were added to the water in which the fish were kept. Therefore, although the amount of iodine accumulated by the thyroid of this anadromous teleost is comparable to that in the shark's thyroid, the rate is somewhat different. The response of iodine uptake in the salmon to excess environmental iodine, and the cellular hypertrophy of thyroids of *Platypoecilus* (Goldsmith *et al.*, 1944) in response to thiourea, indicate a functional pattern in such teleostean thyroids which resembles that of mammals. Olivereau (1949) has pointed out that a further differ-

ence between the selachian thyroid and that of mammals is exemplified in the unresponsiveness of *S. canicula* to treatment with thiourea.

Metabolism of accumulated radioiodine. The synthesis of thyroxine in *S. canicula,* to judge from the radiochromatograms of different post-injection time intervals, follows the same pattern as in mammals. The first I^{131}-marked compounds found were monoiodotyrosine, and diiodotyrosine (at 6 hours), with the monohalogenated compound in excess. Radio-diiodotyrosine then increased in proportion, and the first radio-thyroxine (18.5%) was found 17 hours after injection of the tracer. The absence of radio-thyroxine in the 24-hour sample, though the proportion of new diiodotyrosine is high, is at first puzzling, but an explanation may be offered. The time difference is, after all, quite small. Further, the individual variation we have found to be quite large. Since the 24-hour group seems made up largely of individuals of reduced or retarded iodine uptake (or both), this irregularity in the orderliness of the data possibly can be explained as an expression of this kind of variability. In the 41-hour group the proportion of thyroxine is 33% and at 96 hours radiothyroxine accounts for more than half of the remaining radioactivity in the thyroid. An interesting datum in the 96-hour group is the presence of a small proportion of marked monoiodotyrosine, though marked diiodotyrosine has disappeared. At any rate, the data demonstrate clearly that significant amounts of new thyroxine can be formed less than 17 hours after beginning of accumulation of the tracer in the thyroid. The altering proportions of mono- and diiodotyrosine after 17 hours demonstrate that newly labelled thyroxine continues to be formed for 96 hours or more. It should be borne in mind, however, that in terms of absolute amounts of radiothyroxine, there is much more of this compound present at 17 hours than at 96 hours, even though it comprises a much smaller part of all the I^{131}-labelled compounds at 17 hours.

Histology and radioautography. Although morphological degenerative changes appear to be frequent and more profound in I^{131}-treated thyroids, there seems to be no doubt that to a certain extent thyroidal epithelial degenerative changes are normally found in untreated sharks. Perhaps the most interesting of these features is the shedding of pycnotic, secretion-laden cells into the colloid of the follicular lumen. Such epithelial shedding has been noted in *Scyllium* also by Olivereau (personal communication). The normal frequency of this phenomenon suggests that holocrine secretion is at least one method of release of thyroidal hormone into the thyroidal colloid of *Scyllium.* An interesting counterpart of this process may be seen in the larval lamprey in which whole sheets of iodine-metabolizing cells are shed into the lumen of the endostyle (Gorbman and Creaser, 1942). These instances of shedding of whole cells, and those noted by Olivereau,

lend support to the suggestion made earlier (Gorbman and Creaser, 1942) that holocrine secretion of a thyroglobulin-like substance might be a primitive mode of thyroidal activity.

Greater frequency of pycnotic changes, sharp reduction of mitoses, and the presence of numerous follicles with high, swollen epithelial cells was characteristic of thyroids of I^{131}-injected sharks. Thus it appears that some epithelial radiation injury was produced by the concentration of the radioactive iodine in the thyroidal cells and colloid. If, for purposes of general comparison, it can be assumed that the average 6-hour thyroidal I^{131} accumulation (13.2% of administered dose) represents the approximate maximum isotope accumulation, then this peak produces a concentration of about 0.3 microcuries of I^{131} per mg. of thyroid tissue. In the mouse thyroid, comparable injury was produced by concentrations of about 1.0 microcurie per mg. of thyroid (Gorbman, 1950; Rugh, 1951). Lin and Goldberg (1951) who gave doses of 400 microcuries of I^{131} to the shark *Mustelus californicus*, and the rays *Zapteryx exasperata* and *Holorhinus californicus*, found cytoplasmic vacuolization and nuclear chromatin changes in the thyroidal epithelium. Since they did not give the thyroidal or body weights it is unfortunately impossible to compare our results more closely with theirs.

It is of interest that within as little time as an hour after injection of the tracer iodine, and though the I^{131} uptake at that interval was too small to give more than a very weak radioautograph, the autographed I^{131} was already in the colloid. It was never observed in any of the radioautographs that significant amounts of radioiodine were still in the epithelial cells. Such cellular localization of radioiodine has been noted by Leblond and Gross (1948) in thyroids of iodine-fed rats one hour after administration of I^{131}. We have seen it also in thyroids of normal teleost fishes(*Platypoecilus maculatus, Lebistes reticulatus, Xiphophorus montezumii*) when the animals were fixed after six hours in radioiodine solution (unpublished work). Such rapid accumulation of the I^{131} in the colloid seems to be in agreement with its rapid incorporation into the amino acids and thyroxine. Collodial localization of administered I^{131} was also seen by Lin and Goldberg (1951) in the selachians which they sacrificed 46 hours after injection of the radioisotope.

SUMMARY

Thirty-five sharks, *Scyliorhinus* (*Scyllium*) *canicula* were injected intraperitoneally with tracer doses of carrier free radioiodide. Thyroid glands were taken from animals killed in groups at intervals of 1, 6, 17, 24, 30, 41, 96, and 144 hours after injection. Individual thyroids were measured for gross uptake of I^{131}. Pooled thyroid samples of the different

time intervals were hydrolyzed and analyzed by chromatography. Histological and radioautographic studies were also made of each thyroid gland.

Gross uptake of I^{131} was irregular but rapid, and reached a peak between 6 to 17 hours after injection (maxima: 20% and 34%). Descent from this peak also was rapid. Thyroxine synthesis appeared to follow the same route as in mammals. Monoiodotyrosine and diiodotyrosine were produced first, and a small but significant amount of labelled thyroxine was formed by 17 hours after injection. The proportion of thyroxine to iodinated tyrosines increased with time.

Radioautographs showed the I^{131} to be localized in the colloid very early. Small foci of epithelial cellular necrosis were found, apparently attributable to the concentrated radioactivity.

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THE THYROID AND ADRENAL GLANDS OF CASTRATED C_3H MICE TREATED WITH THIOUREA[1]

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IF THE inbred mouse of Strong's C_3H strain is gonadectomized the adrenal cortex becomes hyperplastic and in the female it produces enough sex hormones to stimulate the uterus and vagina (Woolley and Little, 1945). The morphological alteration of the adrenal is evident as early as thirty-seven days after ovariectomy, and extends to a large part of the cortex in three to six months (Casas, King and Visscher, 1949). If the same strain is given thiourea, the adrenal cortex becomes atrophic (Dalton, Morris and Dubnik, 1945).

The study reported here was undertaken to ascertain the effect on the adrenal cortex of subjecting the C_3H mouse to two apparently antagonistic influences, namely, thiourea to induce atrophy and castration to enhance hypertrophy.

MATERIALS AND METHODS

The animals used in this experiment were inbred C_3H mice from the colony raised at the School of Medicine, School of Tropical Medicine. These were descendants of mice given to the institution by Dr. J. J. Bittner, of the Department of Cancer Biology of the University of Minnesota.

Twenty-five litter-mate male and female mice were started on thiourea at the age of one month, when the animals were weaned. Six males, aged two and one-half months, and six males aged, five months were also placed in this experimental group. The drug was thoroughly blended with finely ground Rockland rat diet so that the concentration of thiourea was 0.3%. The food was given ad libitum, and fresh water was available to the animals at all times. Gonadectomy was performed the day before the special diet was instituted. A group of 25 castrate animals of both sexes was kept on the Rockland diet without thiourea, to serve as controls. An additional control group consisted of 12 intact animals which ingested the drug. All mice, a total of 74 animals, were weighed once a week during the experimental period, which lasted 7 months.

Vaginal smears were made by the lavage method on all females for five consecutive days on alternate weeks. The animals were sacrificed at different intervals beginning at three months after starting the feeding with the goitrogen. Tissues were fixed in Bouin's fluid and stained with Delafield's hematoxylin and eosin.

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RESULTS

Thiourea in the 0.3% concentration was highly toxic to the young mice used in this study. Of the animals started at the age of 1 month, approximately 25% died during the first two weeks of the experiment, in contrast to the high survival rate of the mice operated and started on the stock diet at the same age and under the same environmental conditions. The survival rate was increased by lowering the dose to 0.2% for the first two weeks of the experiment. Some mice disliked the food and refused most of it during the first week so that some of the deaths may have resulted from starvation, particularly among the smaller animals.

The growth rate of the thiourea treated castrates and of the control groups of both sexes is shown in figure 1. These curves show that 0.3% thiourea in the food given ad libitum caused a decreased in the rate of growth in C_3H mice which simulated a condition of hypothyroidism. The rapid growth phase typical of the first month after weaning was not evi-

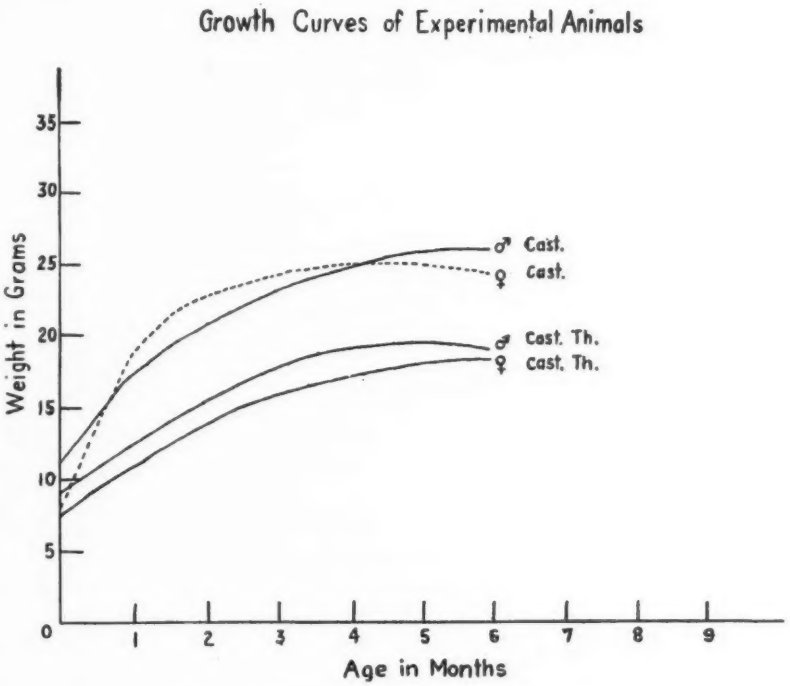

FIG. 1. Growth curves of experimental animals.

dent in the thiourea treated animals, and the average weight of the experimental mice was always less than that of the controls. By the end of six months the thiourea treated animals weighted an average of 6 grams less than the controls, that is, their weight was 25% less than those of the animals that ingested no thiourea. There was definite evidence of cretinism in the animals which ingested thiourea when the weights of all groups were compared 6 months after starting the experiment, even though the shape of the curve was similar in all groups.

The vaginal smear of the control ovariectomized animal four months after operation was a dense mixture of leukocytes, epithelial cells and cornified cells. The cellular content of the smear did not vary much from then on. At times the smear was thinner, and the relative amount of each type of cell changed so that the epithelial cells predominated one day and the leukocytes or cornified cells constituted the greater bulk some other day. A fully cornified smear denoting typical estrus was never encountered. In the castrated females treated with thiourea the smears never showed evidence of vaginal stimulation. At six to seven months after ovariectomy and drug feeding the smears showed occasional cellular elements and frequently contained some large epithelial cells, but the continued dense cellular picture seen in the control animals was never fully developed. The smears of control intact animals fed thiourea showed irregular cycles, and one female twice became pregnant and had a litter. The first litter was born 6 months after treatment was started and the 3 young died after 4 hours. The second litter was born exactly one month later and suffered the same fate.

The thyroid gland of the castrate animals treated with thiourea showed the typical hyperplastic changes described in the intact animals receiving this drug (Dalton, Morris and Dubnik, 1945). A small adenoma was found in an animal that had been castrated and under thiourea administration for seven months. The alternations in the thyroid were about the same both qualitatively and quantitatively after 5 months as after 7 months, with the possible exception of the adenoma mentioned above. Figures 3 and 5 clearly demonstrate the difference between the thyroid of a mouse which ingested thiourea and one which did not.

The adrenals in the castrated controls and in the castrated animals which received thiourea showed no qualitative differences whatsoever. They all contained rather large clear cells with vascuolated cytoplasm which were located in small nest-like groups in the more superficial layers of the cortex. The histological picture was identical with that described by Woolley and Little (1945). In one of these animals the change was more marked than in the others, and in one area a distinct adenoma was beginning to develop. But quantitatively there was a noticeable difference, that is, in the animals

deprived of their gonads but not fed the goitrogen a greater amount of adrenocortical involvement was observed (Figs. 2 and 6) when compared to the castrated, drug treated animals (Figs. 4 and 7). It was interesting to note that the histological changes were always more extensive in the female than in the male. Apparently the adrenal cortex of the female is more sensitive than that of the male to gonadectomy. The control animals that

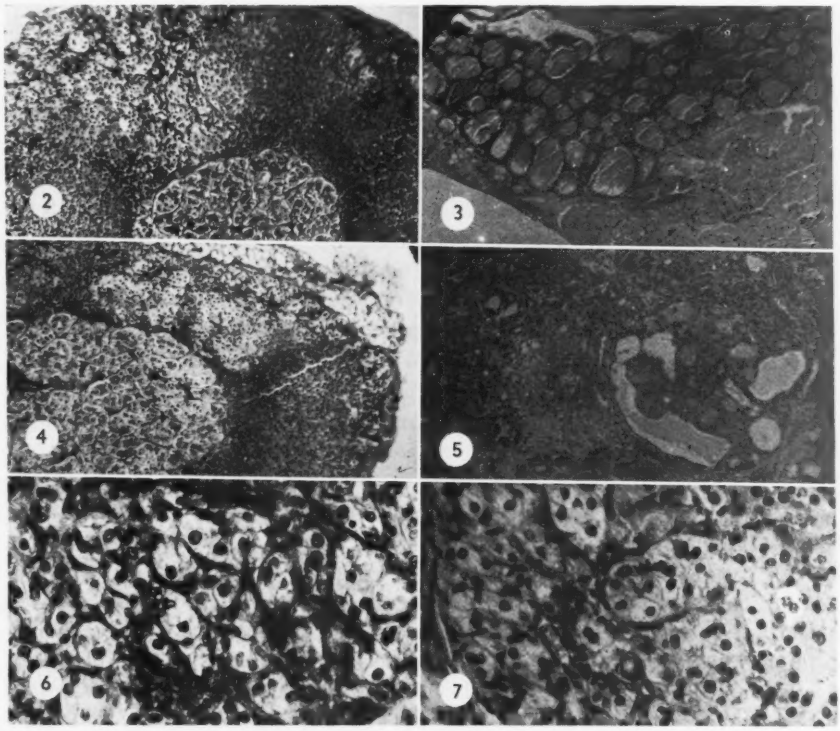

FIG. 2. Adrenal gland of castrate mouse which did not ingest thiourea, six months after gonadectomy. Notice adenomatous process in the cortex with type A and B cells. Magnification, ×80.

FIG. 3. Normal thyroid gland of the animal whose adrenal is illustrated in figure 2. Magnification, ×80.

FIG. 4. Adrenocortical involvement in a castrate mouse which ingested thiourea for six months after gonadectomy. Subcapsular development of the same type of process as shown in figure 2. Magnification, ×80.

FIG. 5. Hyperplastic thyroid of the animal represented in figure 4. Note scanty colloid, obliterated acinar lumina and adenomatous nodule. Magnification, ×80.

FIG. 6. High power view of the process shown in figure 2. Magnification, ×360.

FIG. 7. Another high power view of the process shown in figure 4. Magnification, ×360.

had not undergone castration but had recevied the thiourea did not show any of the above hyperplastic changes.

DISCUSSION

From the above experiments it is evident that 0.3% thiourea in the food given ad libitum caused a decrease in the rate of growth in C_3H mice which was most evident immediately after placing the animals on the experimental diet. Since it was obvious that the mice disliked the thiourea diet, which proved to be rather toxic to the young animals, it might be thought that these results were due to inanition. However Dubnik *et al.* (1950) clearly showed that C3H mice treated with thiouracil consumed more food per unit of body weight, which suggests impared food utilization. In view of these findings it would seem probable that the smaller size of the thiourea treated animals in the present experiment is not due to the reduced food intake. While food consumption was not measured in our experiments, it is possible or even probable that a reduced intake of food did occur at the start of the experiment and had an influence on the degree of toxicity and the rate of growth, but in the later periods it may be assumed that the relation of food consumption to gain in weight was the same with thiourea as without it.

The present experiments confirm once more that the uterus and vagina of the ovariectomized C_3H mouse on a stock diet are stimulated by sex hormones 4 months after removal of the ovaries. Since adrenal hyperplasia was the only striking feature found in these animals, the hormones that stimulated the accessories must have originated from the adrenal cortex. In the experimental group which received thiourea after castration the vagina and uterus were not stimulated. This could be explained on the basis that either not enough hormone was produced by the adrenal tissue and/or the end organ response of the uterus and vagina was so inhibited because of the reduced thyroid hormone, that no evidence of hormonal stimulation could be obtained by the study of vaginal smears. The fact that in the intact animals there was evidence of hormonal stimulation of the uterus and vagina, and ovulation and pregnancy occurred in one animal, in spite of the ingestion of thiourea, speaks against depressed end organ response to ovarian hormones when the ovarian hormones come from the ovary. It is possible that the uterine and vaginal response to ovarian hormones secreted by the adrenal cortex is a different situation which may depend on the quality and quantity of such secretions when they are produced by the hyperplastic suprarenals.

The irregular cycles observed in the intact controls seem to show that 0.3% thiourea in the food is not enough to produce total ovarian atrophy.

The death of the young born to one female of this group was probably due to inability of the mother to suckle the young since they were born alive and died a few hours after delivery. Since no milk was observed in the stomachs of the newborn animals before death it is assumed that lactation was inhibited. Foster nursing was not attempted and therefore it is not known whether the young were able to nurse.

The adrenal cortex of the goitrous castrate showed a subcapsular proliferation of cells with the development of the typical hyperplasia which results after castration in many strains of mice. This finding may be interpreted to mean that the adrenal response to castration in the C_3H mouse is not suppressed by the mechanism set into action by thiourea. However, the hypertrophic changes in the castrate controls on the stock diet were more extensive than in the animals that were fed the drug which indicates that, whatever the effect of thiourea may be, it manifests itself as a reduction in the amount of hyperplastic tissue that develops in the adrenal cortex in response to castration.

The absence of adrenal atrophy during the first 7 months of treatment in the castrated mice provides a wide margin for speculation. The thiourea compounds according to some investigators (Mackenzie and Mackenzie, 1943) (Baumann and Marine, 1945), probably reduce the thyrotrophic, adrenocorticotrophic and gonadotrophic secretions of the hypophysis in intact rats and mice. On the other hand, gonadectomy in the C_3H mouse seems to augment adrenocorticotrophin production, this stimulating the activity of the suprarenals to compensate for the loss of the sex organs by increasing their secretion of sex hormones. Theoretically, we were applying two opposing stimuli in our experiment: One, that of depressing the adrenal cortex indirectly by giving a goitrogen; the other, increasing adrenocortical cutput of sex hormones by gonadectomy. It might be thought that hyperplasia of the adrenal cortex was limited to the cells that secrete sex hormones and was independent of the pituitary, but this is untenable in view of the extensive studies of the pituitary-adrenocortical relationships. The results seem to indicate that gonadectomy is a strong stimulus in spite of the coincidental action of the goitrogen. The absence of estrogens in the female castrate probably releases the pituitary from a certain amount of inhibition which results in an increased production of pituitary factors, especially, gonadotrophins and adrenocorticotrophin, which in return prevent or delay the adrenal atrophy that results from treatment with the goitrogens in the intact animal. These trophic hormones may have led to the adrenal hypertrophy which occurred though slightly delayed, in the castrate animal treated with thiourea, and which is so typical of the castrate C_3H mouse on a plain stock diet.

SUMMARY

Gonadectomy of C_3H mice of both sexes immediately followed by continuous ad libitum feeding with thiourea (0.3%) in the stock diet, yielded the following results after 6–7 months:

1. The weight of the experimental mice was less, by 25%, than that of the castrate controls.
2. Vaginal smears detected sex hormone stimulation 4 months after gonadectomy in the control females, but no signs of this effect appeared after castration and drug treatment in the experimental groups.
3. Histological study of the thyroid showed hyperplastic changes and diminished production of colloid secretion.
4. The adrenal cortex showed a hyperplastic process limited to the outer region of the cortex in which there appeared large cells with clear cytoplasm surrounded by small, darkly staining cells. The alterations seem to develop earlier and become more extensive in the female than in the male mice.
5. The adrenal atrophy which has been reported in mice and rats after giving thiourea was prevented in C_3H mice by gonadectomy for 6 to 7 months. Contrary to expectations, during this time the adrenal glands in the gonadectomized animals develop hyperplastic changes. The explanation of this finding is not entirely clear but it seems probable that the castration effect is not suppressed by the goitrogen when both stimuli are present for at least this length of time.

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A DIFFERENCE BETWEEN THE EFFECTS OF DIETARY CALORIE RESTRICTION ON THE ESTROUS CYCLE AND ON THE 24-HOUR ADRENAL CORTICAL CYCLE IN RODENTS

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INTRODUCTION

THE presence of a physiological rhythm in the numbers of circulating eosinophils in intact animals including man and the absence of this rhythm in adrenalectomized humans have been reported (Halberg and Visscher, 1950, Halberg, 1951, Halberg *et al.* 1951). This report presents studies of the 24-hour changes in numbers of eosinophils in the mouse restricted in calories for several months. If, as seems likely, the 24-hour changes in eosinophils are due to changes in the output of adrenal cortical hormone, these results have a bearing on the question of whether such hormone output is influenced by caloric restriction. A comparable steroid rhythm, namely that responsible for the estrous cycle, is inhibited by undernourishment in animals and in humans (Loeb, 1921, Papanicolau and Stockard, 1920, Huseby *et al.* 1945). The finding of a marked 24-hour periodicity in eosinophil levels of underfed mice appears to be of interest in view of the limiting effect of dietary caloric restriction on the development of tumors.

MATERIALS AND METHODS

C_3H mice of the Z stock were employed in this investigation. From weaning they were singly housed in $8'' \times 6'' \times 6''$ sheet metal cages with a fine wire-mesh front. They were kept in a temperature-controlled room at $78 \pm 2°$ F. The room was illuminated by artificial light. A time-switch turned lights on at 06:00 and off at 18:00. The mice had free access to water throughout the study. They were fed special diets (Table 1) from weaning. The composition of the diets was such that the intake of the various con-

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TABLE 1. COMPOSITION OF DIETS

	Control (gm.)	Restricted (gm.)
Crude Casein	28.9	48.8
Glucose	34.0	8.7
Lard	17.5	9.4
Brewer's Yeast	8.3	13.9
Alfalfa Meal	4.1	6.9
Salt Mixture*	4.1	7.0
Cod Liver Oil	2.1	3.5
Wheat Germ Oil	1.0	1.8
Total	100.0	100.0

* Salt Mixture—Jones-Foster Formula (1942) parts by weight, NaCl—585, KH_2PO_4—1633, $MgSO_4$—241, $CaCO_3$—1602, $FeSO_4$—62, KI—3.32, $MnSO_4$—16.9, $ZnCl_2$—1.09, $CuSO_4$—1.28, $COCl_2$-$6H_2O$—0.095.

stituents by the mice on calorie restriction differed from that of the full fed controls only in the reduced carbohydrate and fat ingested. The calorie restricted mice were fed 1.51 gms and the controls 2.66 gms per day. Both diets were offered at 18:30. This time of day was chosen in view of the predominantly nocturnal feeding habits of mice with continuous access to food. Tail blood was used for counting eosinophils. The procedures employed for this purpose have been described (Halberg *et al.*, 1951b). Counts were obtained at various times of day over a period of one month. At least four days elapsed between two successive observations on a single animal. The animals were six months old when the first counts were obtained. The average body weight of the control mice at this time was 29 gm., as compared with an average body weight of 18 gm. of the restricted mice.

RESULTS

All the counts obtained throughout this investigation, expressed as per cent of the mean count for each mouse, are presented in Table 2, tabulated for the period of the day but irrespective of the day on which they were obtained. The counts show considerable scatter in individual values at specific times of day but there are obvious trends in the values at different time periods. It should be noted that observations on individual mice show considerable variability in times of maximum and minimum counts.

It can be seen from Table 3 that the differences in mean numbers of eosinophils during the middle morning hours as compared with the early evening hours are very marked for both groups of mice. The observation of an evening "low," as well as that of a morning "high," is in keeping with earlier observations on fullfed mice of different strains (Halberg and Visscher, 1950). The relative eosinophil levels of eight control mice and of eight restricted mice with complete data and their means are graphed in Figure 1. It can be seen that the times of the maximum and the minimum in mean number of eosinophils are roughly the same for both groups of mice. An analysis of variance (Snedecor, 1946) on these data reveals that the differences in eosinophil levels noted at the different peri-

TABLE 2. RELATIVE EOSINOPHIL LEVELS* IN AD LIBITUM FED AND IN CALORIE RESTRICTED MICE

Mouse No.	Control Mice									
	Time of sampling**									
	1	2	3	4	5	6	7	8	9	10
1	115	74	65	89	268	159	105	38	81	6
2	165	47	82	151	156	141	10	121	106	21
3	64	78	76	269	166	154	84	14	83	11
4	39	128	95	283	80	189	53	73	41	19
5	39	116	232	249	116	120	64	17	41	6
6	71	80	80	243	100	55	55	262	25	29
7	48	70	170	286	190	90	29	70	27	20
8	174	139	29	218	93	106	58	42	77	64
9	115	87	58	162	91	166	—	140	76	5
10	57	115	182	—	326	71	65	13	52	19
11	—	156	111	—	87	140	—	153	32	21
12	—	37	82	370	138	128	—	5	30	10
13	—	51	65	352	181	51	—	46	24	30
14	—	75	235	140	82	148	—	30	40	50
15	56	43	130	—	211	178	82	108	63	29
16	126	58	129	220	94	136	106	—	26	5
17	85	75	101	202	166	—	87	55	29	—
18	84	118	169	153	123	—	7	79	39	128
19	—	72	244	132	129	139	—	46	20	20
20	—	103	143	179	161	86	—	46	42	40
21	—	—	—	319	—	35	—	19	27	—
22	—	201	78	273	118	59	—	0	44	27
23	—	68	117	310	225	42	53	43	36	6
24	72	204	155	—	—	72	16	155	—	26
25	157	91	49	302	80	120	30	35	—	36
26	53	116	210	218	100	72	—	—	49	—
27	—	137	176	184	161	88	—	15	0	39
28	—	76	135	281	58	95	—	—	37	18

Mouse No.	Restricted Mice									
	Time of sampling**									
	1	2	3	4	5	6	7	8	9	10
1	61	183	159	244	203	86	12	16	14	21
2	59	202	101	201	175	122	35	31	35	39
3	146	195	104	214	227	16	0	0	40	58
4	115	166	166	240	166	48	5	9	27	58
5	144	83	243	241	170	47	9	14	21	28
6	11	169	81	260	191	154	76	16	26	16
7	28	118	183	379	55	132	46	0	38	21
8	31	55	192	382	127	96	0	28	48	41
9	33	229	93	424	28	23	27	5	—	38
10	—	25	—	390	53	—	0	17	—	115
11	—	157	75	395	123	34	0	0	96	20
12	—	43	78	384	87	176	—	11	7	14
13	—	28	—	—	192	294	50	6	53	77
14	107	82	151	221	213	54	0	3	69	—
15	132	56	90	515	13	0	5	10	—	79
16	0	106	257	—	189	287	—	7	18	140
17	46	71	78	192	263	178	28	0	—	44

* Expressed as per cent of the mean count for each mouse.

** The numerals listed at the heads of the columns in this section designate periods of sampling as set forth in column 1 of Table 3 below.

ods of sampling are significant below the one per cent level for both groups of mice. The analysis of variance summary follows:

Group	D.F.	Variance	F
Control	9	25,066	8.13**
Restricted	9	54,348	24.23**

Furthermore, Figure 1 suggests that the amplitude of the eosinophil rhythm is slightly greater in restricted than in control mice. It seemed therefore worthwhile to test the hypothesis that the pattern of true means (as graphed against time in Fig. 1) is the same for the restricted group and for the control group. This hypothesis of "no time-group interaction is equivalent to the hypothesis that the two patterns of means in Fig. 1 are random deviations from the same true pattern. Rejection of the "no interaction" hypothesis requires rejection of the possibility that the differences in pattern, shown in Fig. 1, are random chance differences. This in turn imposes the conclusion that real pattern differences are present. The F value obtained for the time-group interaction is significant below the one per cent level ($.005 < P < .01$). It appears therefore that the small incre-

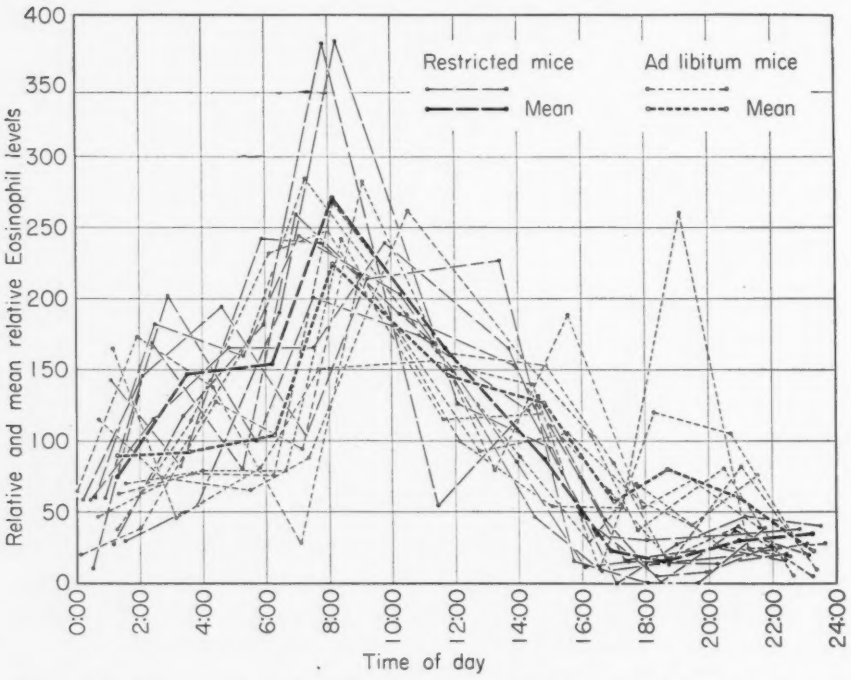

FIG. 1. The 24-hour eosinophil rhythm in calorie restricted and in ad libitum fed mice.

TABLE 3. MEAN RELATIVE EOSINOPHIL LEVELS (MREL) AND THEIR STANDARD DEVIATIONS (SD) IN (A) THE CONTROL GROUP AND (B) THE RESTRICTED GROUP

Period of sampling	Control mice			Restricted mice		
	No.	MREL	SD	No.	MREL	SD
1. 00:28–02:27	17	89	45	13	70	52
2. 02:04–06:21	27	97	43	17	116	67
3. 05:13–08:16	27	126	60	15	137	61
4. 06:54–10:48	23	233	76	15	312	101
5. 10:12–14:26	26	142	64	17	146	74
6. 13:50–16:27	26	109	44	16	97	77
7. 15:57–18:22	16	57	32	15	20	23
8. 17:19–20:32	25	65	61	17	10	9
9. 20:07–22:31	26	44	24	13	38	24
10. 22:10–01:09	25	27	26	16	51	36

ment in the amplitude of the eosinophil rhythm in restricted mice over that of control mice is statistically significant. The main point of this paper, however, is that the amplitude is not less.

Certain organ weights were also obtained in these two groups of mice, upon completion of the investigation on number of eosinophils. Relative organ weights were computed by expressing each of the absolute values as per 100,000 of body weight. The mean relative uterine horn weights were 195.6 ± 23.0 for the controls and 39.8 ± 1.4 for the restricted mice ($P < .001$). For ovarian weights the comparable values were 10.3 ± 1.0 and 8.1 ± 0.6 ($P = .206$). By contrast, the mean relative adrenal weights were 9.3 ± 0.6 for the controls and 13.1 ± 1.2 for the restricted mice ($P < .001$). It is therefore apparent that the adrenal glands were not atrophied in calorie restricted mice, whereas the uteri show the expected and previously observed selective atrophy.

DISCUSSSION

The maintenance of the eosinophil rhythm in calorie underfeeding in intact mice indicates that the mechanism underlying that rhythm is a persistent and fundamental phenomenon. This mechanism is probably associated with some of the major 24-hour rhythms found in mammals, especially with the alternation of rest and activity. The literature on activity rhythms has been reviewed by Calhoun (1944, 1945, 1946). Studies by Johnson (1926, 1939) and more recently by Browman (1952) indicate that illumination and temperature, among other factors, determine the timing of the daily maximum and minimum in the activity of rodents. Data from this laboratory show that changes in the timing of the maximum and minimum in number of eosinophils of mice can be brought about by changes in illumination, within 7 days, and also, more slowly, by changes in feeding time (Halberg and Visscher, 1952b). Patients with bilateral adrenalectomy or with Addison's disease fail to exhibit the 24-

hour eosinophil rhythm (Halberg *et al.* 1951a, 1951c), indicating that the phenomenon depends upon a functioning adrenal cortex. In view of the 24-hour periodicity noted for 17-ketosteroid and neutral reducing lipid excretion in the urine (Pincus, 1943, Pincus *et al.* 1948) and for the number of circulating lymphocytes (Elmadjian and Pincus, 1946), it appears probable that adrenal cortical function normally exhibits 24-hour periodicity. Since the 24-hour rhythm in numbers of eosinophils persists in underfeeding it can be suggested that the 24-hour adrenal cortical cycle is not suppressed under circumstances known to suppress the ovarian estrous cycle. Studies in this laboratory on the same strain of mice kept on identical diets have shown that the degree of calorie restriction here employed suppresses the estrous cycle (Puh-Lee and Visscher, 1952). It may be that the adrenal cortical cycle is either (a) not controlled by the hypophysis, or (b) if so controlled, that pituitary adrenal-cortical function is not abolished and not even decreased by the same degree of dietary calorie restriction, which eliminates gonadotropin production in C_3H mice, as well as in other animals (Marrian and Parkes, 1929). Since it has been found that in panhypopituitarism in man the 24-hour rhythm in eosinophil levels is also lost (Halberg *et al.* 1951a, 1951c), and since the adrenal of the underfed mice investigated did not atrophy, it can be inferred that the effects of calorie restriction on the pituitary are not the same as to gonadotropic and adrenotropic functions (Boutwell *et al.* 1948). But whether the mechanism ultimately responsible for the 24-hour eosinophil rhythm of underfed mice is assumed to be (a) the adrenal gland only, responding to changes in corticoid levels at the periphery, or, perhaps, (b) a pituitary-adrenal rhythmic activity, the data support the hypothesis of a functioning adrenal cortex under the circumstances of a 50 per cent restriction in dietary calories without protein, vitamin or mineral restriction.

SUMMARY

Pituitary and/or adrenal cortical function was investigated in two groups of female C_3H mice by means of the evaluation of their 24-hour periodicity in eosinophil levels. A regular physiological rhythm in the numbers of circulating eosinophils was observed in calorie-restricted as well as in full-fed mice. The maintenance of the 24-hour adrenal cortical cycle under circumstances associated with the suppression of the estrous cycle is demonstrated. The significance of this finding is discussed.

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THE ADRENAL CORTEX OF THE PANTOTHENIC ACID DEFICIENT RAT: MODIFICATION OF THE LESION BY ACTH AND CORTISONE TREATMENT,[1,2]

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INTRODUCTION

MANY workers have observed the adrenal lesion accompanying pantothenic acid deficiency in rats (Daft and Sebrell, 1939; Nelson, 1939; Ashburn, 1940; Deane and McKibbin, 1946). Briefly the lesion consists of congestion of the inner cortical zones with hemorrhage, necrosis, fibrosis, hemosiderin deposition and calcification. The medulla and zona glomerulosa are rarely involved.

However, the mechanisms involved in the development of this lesion have received little attention. Adrenal cortical extract, desoxycorticosterone and anterior pituitary extract failed to modify the adrenal lesion in pantothenic acid deficient weanling rats (Mushett and Unna, 1941). Unpublished experiments of a similar type in our own laboratory were likewise unsuccessful. In the course of these experiments it occurred to us that two things might be needed—first, larger doses of more highly purified hormone preparations and, second, a more uniformly severe deficiency state in our rats. The latter became available with the technique described by Nelson, *et al.* (1950). After these two factors had been taken into account and proper formulation of the experiments here reported, we were able to modify in significant degree the adrenal lesion. These findings, coupled with previous evidence suggestive of a functional adrenal insufficiency in the pantothenate deficient rat (Gaunt, *et al.* 1946; Dumm, *et al.*, 1949; Winters, *et al.*, 1952a, b, c) have led us to a concept of the mechanisms involved in the development of "hemorrhagic necrosis" of the adrenal.

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[2] We are indebted to Dr. Irby Bunding of Armour and Company for generous gifts of ACTH; to Merck and Company for cortisone acetate; to Dr. Dwight J. Ingle of Upjohn and Company for a generous supply of Lipoadrenal Extract; and to Hoffmann La-Roche and Abbott Companies for vitamins.

METHODS

General Considerations. The technique used in obtaining severely deficient young rats (Nelson, *et al.*, 1950) consisted essentially of the following: Lactating rats were placed on a pantothenic acid deficient diet immediately after littering. Young rats which had suckled from such mothers were then weaned to the deficient diet. This intriguing technique produces a uniformly severe deficiency state, probably from taking advantage of the larger requirement for pantothenic acid of the growing animal as compared to that of the more mature animal (Unna and Richards, 1940). This fact has recently been reemphasized by Lotspeich (1950), who has shown that the mature animal, ordinarily notoriously difficult to render deficient by feeding a deficient diet alone, may be made so by administration of growth hormone in addition to feeding a deficient diet.

Animals, diets and conditions. All rats employed were of the Sprague-Dawley strain. The young rats were nursed from mothers receiving either a deficient or a control diet and were weaned on the 21st day to a corresponding deficient or control diet. The composition of the diets and the experimental conditions are the same as have been described previously (Winters, *et al.*, 1952a).

Drugs. Purified ACTH (Armour) was diluted in sterile normal saline and injected intraperitoneally. After weaning, the rats treated with ACTH received an average daily dose of 4.0 mg. A saline suspension of cortisone acetate was injected subcutaneously in the amount of 2.0 mg. per day. Aseptic technique was employed in all injections.

Pathology. All rats were sacrificed with a sublethal dose of nembutal and exsanguination from the heart. Tissues were quickly removed, cleaned of fat, weighed and then fixed in neutral buffered formalin. The adrenals were stained with hematoxylin and phloxine B and tested for cholesterol by a modification of the Liebermann-Burchardt reaction (Glick, 1949).

EXPERIMENTAL RESULTS

At weaning, all of the animals of the deficient groups showed a retarded weight gain but no other signs of the deficiency (see Table 1). As the experiment progressed, however, clinical evidence of a severe pantothenic acid deficiency became abundantly clear with diarrhea, "burning feet," "porphyrin whiskers" and crusting of the eyes. It is of special interest to note that in the cortisone-treated deficient group these signs were all present, and, if anything, were more prominent than in either the ACTH treated or untreated deficient groups. Thus, the deficient animal even when protected from adrenal insufficiency by exogenous cortisone therapy will demonstrate the signs of depletion of the vitamin from the peripheral tissues.

The control animals in all groups showed no abnormal external signs except those in the cortisone treated group, which failed to gain weight as rapidly as either the ACTH or untreated groups.

Pathology. The pathology of the untreated groups is as follows: The adrenals of the untreated deficient group were enlarged (89.1 mg. per cent) and mottled. Microscopically, these adrenals showed necrosis principally involving the zona fasciculata with a variable amount of necrosis in the reticularis and and glomerulosa (Fig. 1, G. and H.). The cholesterol con-

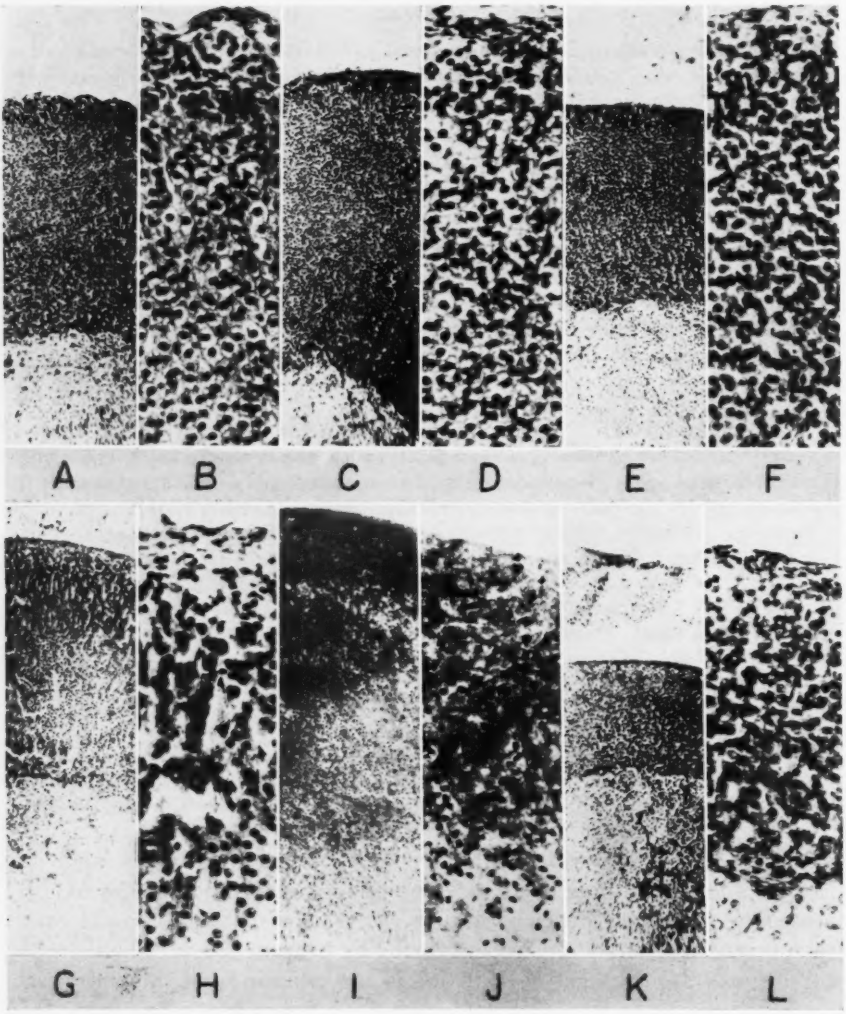

FIG. 1. Representative photomicrographs of adrenals, untreated, ACTH- and cortisone-treated, of control and pantothenic acid deficient rats.

Adrenals pictured are from animals surviving the entire experimental period. All sections have been fixed in formalin, imbedded in paraffin and stained with hematoxylin and phloxine B.

A. Low power (100×) view of untreated control.

B. High power (440×) view of A.

C. Low power view of ACTH treated control.

tent varied inversely with the extent and severity of the lesion. Thus, in the most severely affected glands there was only a small amount of cholesterol in the glomerulosa or none at all. Adrenals from untreated control rats were normal as to size (33.1 mg. per cent) and morphology Fig. 1, A and B).

The pathology of the ACTH treated groups is as follows: Grossly the ACTH treated deficient adrenals were very large (158.5 mg. per cent) and solid black in color. Microscopically, the magnitude and severity of the lesion were greatly accentuated (Fig. 1, I and J). In these animals the necrosis of cortical cells included not only the inner zones but the glomerulosa as well. In several instances the medulla was also noted to be involved to a variable degree by hemorrhage and necrosis. The hemorrhage into the medulla occurs in the glands most severely affected and is probably secondary to that in the cortex by virtue of a common blood supply. The cholesterol content of these glands was virtually nil. Capsular "adenomas," of the type observed by Selye and Stone (1950) in *normal* ACTH treated rats were observed in the severely damaged adrenals of deficient ACTH treated rats (Fig. 2, A and B). One such "adenoma" was observed in an untreated deficient adrenal, but none were observed in any of the control groups. ACTH treated control adrenals showed some increase in weight (43.5 m. per cent) and excessive storage of cholesterol, but no hemorrhage or necrosis (Figure 1, C and D).

The pathology of the cortisone treated groups is as follows: The adrenals of the cortisone treated deficient animals, in contrast to those in the above two deficient groups, were smaller than normal (32.0 mg. per cent), pink in color and entirely free of the histological stigmata of the deficiency as revealed by serial sections of these glands (Fig. 1, K and L). Cholesterol in such glands was confined to the outer zone. If the deficient rats were treated for at least seven days with cortisone their adrenals were identical with those of the cortisone treated control animals as to size (26.5 mg. per cent) and morphology (Fig. 1, E and F).

D. High power view of C.
E. Low Power view of cortisone treated control.
F. High power view of E.
G. Low power view of untreated deficient.
H. High power view of G.
I. Low power view of ACTH treated deficient.
J. High power view of I.
K. Low power view of cortisone treated deficient.
L. High power view of K.

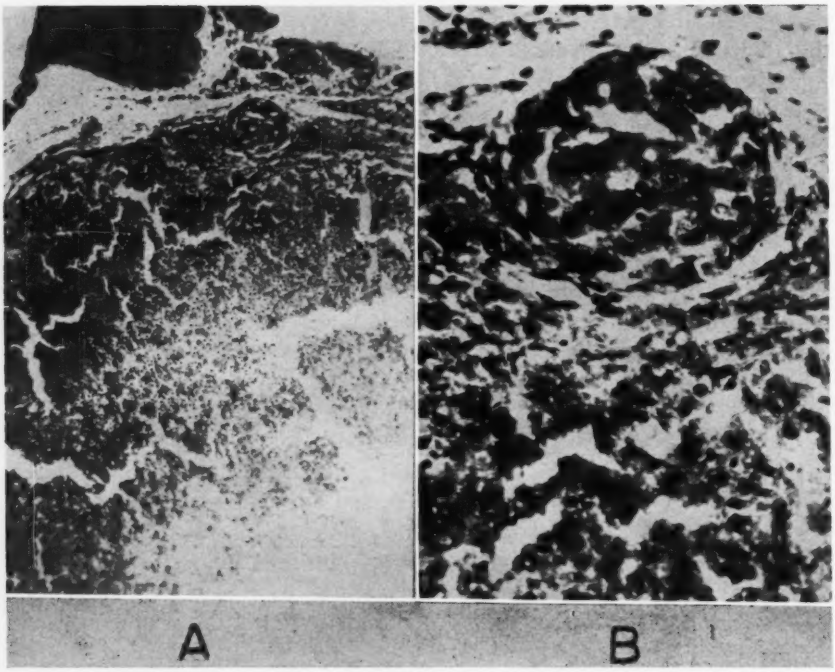

FIG. 2. Views of intracapsular "adenoma" in the adrenal of an ACTH treated pantothenic acid deficient rat.

A. Low power (100×) view showing extensive hemorrhage, necrosis of cortical cells and capsular "adenoma."

B. High power (440×) view of the capsular "adenoma."

DISCUSSION

It would appear from the above results that the lesion of "hemorrhagic necrosis" in the pantothenic acid deficient rat can be completely prevented by giving large doses of cortisone and can be markedly accentuated by giving large doses of ACTH. These results suggest that ACTH is the main factor involved in the production of the adrenal lesion since exogenous ACTH either reinforces or supplants the animal's own ACTH and since cortisone blocks the secretion of this trophic hormone (Sayers, 1950). It now remains to intergrate these results with the natural history of "hemorrhagic necrosis." The essential points of this hypothesis are presented in Fig. 3.

During the early phases of the deficiency, the initially high level of Coenzyme A (Kaplan and Lipmann, 1948), the functional form of pantothenic acid in metabolism (Lipmann, *et al.*, 1947) is rapidly depleted in

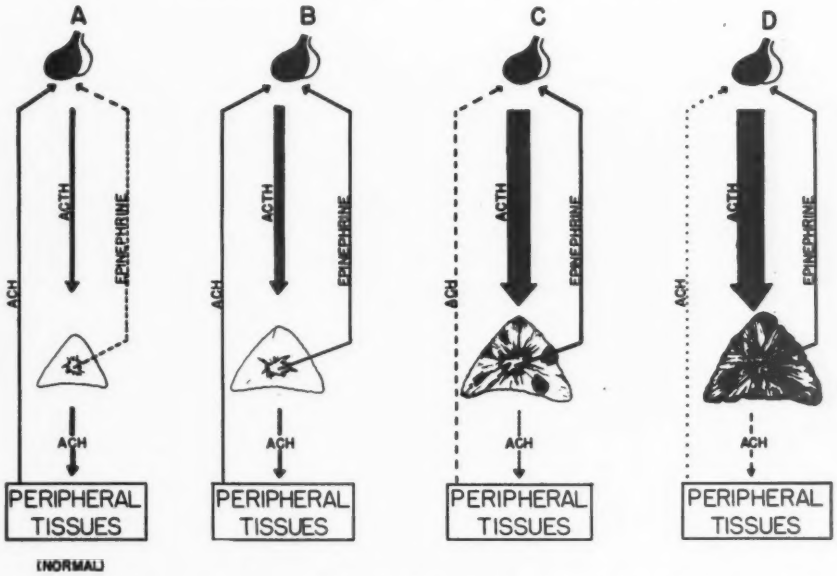

FIG. 3. A schematic representation of the natural history of the adrenal lesion produced by pantothenic acid deficiency in the rat. B, C, and D represent the increasing hemorrhage and necrosis of the adrenal over the period of the deficiency. Attention is drawn to the increasing ACTH and to decreasing ACH production associated with the development of the lesion.

the adrenal cortex of the rat (Olson and Kaplan, 1948). As the depletion progresses a functional adrenal insufficiency develops which with time becomes more marked. This is probably due not only to further depletion of the vitamin, but also to an increased demand for cortical hormone incident to non-specific stresses imposed by the dietary deficiency. In this situation, as the circulating level of cortical hormone drops, the anterior pituitary is stimulated to increased secretion of ACTH (Sayers, 1950). Thus, hypertrophy of the cortex is an early sign of this deficiency (Deane and McKibbin, 1946). As the deficiency progresses, the adrenal becomes increasingly unable to accelerate its rate of secretion of hormone in response to the elevated level of ACTH production. A vicious cycle is thereby initiated analogous to the situation seen in the thyroid gland when treated with certain goitrogens. We believe, therefore, that the sustained high level of circulating ACTH acting upon the infeebled gland produces, directly or indirectly, "hemorrhagic necrosis" of the adrenal cortex. In this connection it is interesting to note that Ingle (1951) has produced a lesion not unlike that seen in pantothenic acid deficiency by treating *normal* rats (with one adrenal enucleated) with large doses of ACTH.

There are 2 important considerations in the acceptance of this theory. The first revolves about the role of pantothenic acid in adrenal metabolism. This has been discussed in some detail by Cowgill, *et al.* (1952), who concluded that the most likely possibility is that pantothenic acid (as Coenzyme A) catalyzes the synthesis of cholesterol from acetate units. In the presence of a functionally insufficient adrenal gland a reduction of adrenal cholesterol as demonstrated histochemically in the present study

TABLE 1. SUMMARY OF THE EXPERIMENTAL DATA

Group	Number of animals	Hormone treatment	Ave. body wt. in grams†		Ave. adrenal wgt. in mg. %
			Initial	Final	
Deficient	12	None	39 ± 1.5	39 ± 2.4	89.1 ± 10.0
Deficient	12	ACTH	37 ± 1.7	36 ± 2.0	158.5 ± 17.9*
Deficient	16	Cortisone	23 ± 0.4	24 ± 1.0	32.0 ± 2.6*
Control	6	None	52 ± 1.6	67 ± 1.9	33.1 ± 2.1
Control	6	ACTH	48 ± 0.8	67 ± 2.7	43.5 ± 0.8
Control	6	Cortisone	47 ± 2.0	44 ± 2.2	26.5 ± 0.3**

All data is expressed as the mean ± the standard error.
† Initial weight = Average weight when weaned. Final weight = Average weight when sacrificed.
* Indicates statistical significance when compared with adrenal weights from untreated deficient rats. ($p = <0.01$).
** Indicates no statistical significance when compared with adrenal weights from cortisone treated deficient rats. ($p = >0.20$).

as well as chemically (Winters, *et al.*, 1952c) implies a decrease in the rate of synthesis rather than an increase in the rate of utilization.

Second, if the factor producing cortical exhaustion is ACTH, a functionally intact anterior pituitary gland is implied. In the absence of normal adrenal function adequate tests for pituitary (ACTH) function are exceedingly difficult. Unpublished observations in this laboratory, however, reveal no gross or histological abnormalities in the pituitaries of deficient rats. Indirect evidence of a functionally intact pituitary in this deficiency state is shown in the sparing action of cortisone as seen in this study and in hypertrophy of the adrenal cortex (Deane and McKibbin, 1946).

SUMMARY

1. The use of very young rats yields more uniform and more easily reproducible adrenal lesions in pantothenic acid deficiency.

2. Adrenal damage of pantothenic acid deficiency in such animals may be greatly magnified by treatment with ACTH. Exogenously administered cortisone prevents the development of the characteristic adrenal lesion.

3. Cholesterol demonstrable in the adrenal cortex of deficient rats may be reduced to the vanishing point by treatment with ACTH. Deficient rats adequately treated with cortisone reveal an adrenal cholesterol, the

amount and distribution of which is identical with that in similarly treated controls.

4. A possible explanation is offered for the natural history of "hemorrhagic necrosis" of the adrenal in pantothenic acid deficiency in the rat.

ACKNOWLEDGMENTS

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NOTES AND COMMENTS

REPRODUCTIVE PERFORMANCE IN EXTREMELY HYPOTHYROID MALE GUINEA PIGS[1]

Following frequent injections of thyroxine into 6 male guinea pigs and surgical thyroidectomy of 14, hyper- and hypothyroid animals were produced in which the range of oxygen consumption was 48.1 ± 1.84 to 80.1 ± 3.75 cc/100 gm/hr., and heart rate was 241 ± 11.0 to 332 ± 5.2 beats/min. (Young, Rayner, Peterson and Brown, 1952). Reproductive performance within this range was tested. The strength of sex drive, the size of litters born to normal females mated with the experimental males, the per cent of pregnancies terminating 66 days or later, and the per cent of young born alive were not significantly different from these indices of reproductive capacity in the controls. The per cent of fertile matings, on the other hand, was somewhat lower following thyroidectomy (96.3% to 85.5%), although some of the males having a low thyroid activity were completely fertile. The suggestions were made 1) that no close relationship exists between the thyroid and reproduction in the male guinea pig unless it is in the fertility of the matings, 2) that the range of thyroid activity within which reproduction can occur is wide, and 3) that individuals in their reproductive performance react differently to low levels of thyroid hormone.

A further suppression of the rate of oxygen consumption from 48.4 ± 1.4 to 37.3 ± 1.2 cc./100 gm./hr., and heart rate from 248.0 ± 17.5 to 190.2 ± 7.4 beats/min. was accomplished by the administration of 400μ. C. I^{131} to 8 of the 14 surgically thyroidectomized males used in the earlier experiment. Seven of the 17 control animals were retained. Their average oxygen consumption had decreased from 52.4 ± 6.0 to 48.9 ± 5.5 cc./100 gm./hr., and heart rate increased from 258.3 ± 16.1 to 260.0 ± 8.0 beats/min.

Within the experimental group there was no change in reproductive performance that was clearly different from that in the control group except in the per cent of fertile matings and fertile artificial inseminations. The latter were performed with spermatozoa removed from the epididymides after the last mating test. In the experimental group 65.9% of 44 matings and inseminations were fertile whereas in the control group 86% of 43 were fertile. The average strength of sex drive based on 150 tests was 7.2 ± 0.4 in the experimental group and 7.4 ± 0.3 in the control group. Average litter size in the two groups was 3.1 and 3.0; the per cent of pregnancies terminating 66 days or later was 100 and 91.9; and the per cent of young born alive was 92 and 84.8, respectively.

Most striking was the difference in the performance of individuals. None of 6 matings was fertile in one male in which oxygen consumption was 38.5 cc./100 gm./hr. and heart rate 198 beats/min., but 6 of 6 matings were fertile in another male in which the measures of thyroid activity were almost equally low, oxygen consumption being 38.0 cc./100 gm./hr. and heart rate 208 beats/min.

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The data strengthen the suggestion that hypothyroidism in the male lowers the per cent of fertile matings, but they also indicate that the range of thyroid activity within which reproduction is possible is lower, and the difference in the reaction of individuals to hypothyroidism is greater than we concluded when the other article (Young, Rayner, Peterson and Brown, 1952) was prepared.

For the problem of the relationship between the thyroid and reproduction in the male, the data suggest the working hypothesis that there is variation from species to species and from individual to individual. There may be species or individuals in which the range of thyroid activity compatible with reproduction extends from a relatively high degree of hyperthyroidism to a relatively low degree of hypothyroidism. The male guinea pig would seem to fall in this category. There may be other species in which the limits within which reproduction can occur is narrower and it may lie in the middle of what we refer to as the spectrum of thyroid activity, or, it may lie toward either end. Reproduction in a species or individual dependent on a high level of thyroid activity might be adversely affected by a change toward hypothyroidism, whereas the converse might be true for a species or individual normally functioning at a relatively low level. Experiments suggested by this hypothesis could lead to a clarification of the conflicting experimental and clinical data.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send FOUR COPIES of the title and abstract to the Vice-President, Dr. Lawson Wilkins, Johns Hopkins Hospital, Baltimore 5, Maryland, not later than FEBRUARY 1, 1953. IT IS IMPERATIVE THAT THE ABSTRACTS BE INFORMATIVE AND COMPLETE WITH RESULTS AND CONCLUSIONS IN ORDER THAT THEY MAY BE OF REFERENCE VALUE AND SUITABLE FOR PRINTING IN THE PROGRAM AND JOURNALS OF THE SOCIETY. THE FOLLOWING REGULATIONS FOR THE PREPARATION OF ABSTRACTS AND TITLES MUST BE CAREFULLY FOLLOWED TO INSURE CONSIDERATION OF THE PAPER FOR THE PROGRAM:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-

authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

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Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was

unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.